Blood Science

Hematopoietic stem cell heterogeneity in non-human primates revealed by five-lineage output bias analysis

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Abstract

Understanding hematopoietic stem cell (HSC) heterogeneity is crucial for treating malignant blood disorders. Compared with mice, we have limited knowledge of the heterogeneity of human HSCs. Fortunately, non-human primates (NHPs) have become the best animal models for studying human HSCs. Here, we employed a public dataset derived from NHP autologous bone marrow transplantation, and focused on a total of 820 HSC clones with reconstitution capacity of all available five lineages (granulocyte, monocyte, B cell, T cell, and natural killer cell) at two time points (11/12 and/or 42/43 months). Intriguingly, unsupervised clustering on these clones revealed six HSC subtypes, including a lymphoid/myeloid balanced (LM-balanced) subtype and five single-lineage–biased subtypes. We also observed that the subtypes of these HSC clones might change over time, and a given subtype could transition into any one of the other five subtypes, albeit with a certain degree of selectivity. Particularly, each of the six subtypes was more likely to turn into lymphoid-biased rather than myeloid-biased ones. Additionally, our five-lineage classification method exhibited strong correlation with traditional lymphoid/myeloid bias classification method. Specifically, our granulocyte- and monocyte-biased subtypes were predominantly attributed to α-HSCs, while LM-balanced, B cell-biased, and T cell-biased subtypes were primarily associated with β-HSCs. The γ-HSCs were composed of a small subset of B cell-biased and T cell-biased subtypes. In summary, our five-lineage classification identifies more finely tuned HSC subtypes based on lineage output bias. These findings enrich our understanding of HSC heterogeneity in NHPs and provide important insights for human research.

Key Words: Clonal analysis; Dynamic changes; Hematopoietic stem cell; Heterogeneity; Non-human primate

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1. INTRODUCTION

Hematopoietic stem cells (HSCs), a rare cell population at the apex of the hematopoietic hierarchy, possess self-renewal and multilineage differentiation capabilities, enabling the production of nearly all blood cell lineages.[1] Accumulating mouse studies utilizing various techniques (eg, refined cell sorting, limiting dilution, single-cell transplantation, genetic lineage tracing, single-cell lineage tracing)[1–3] have unveiled heterogeneities within HSC populations in terms of cell surface marker phenotype,[4] cell cycle status,[5] reconstitution kinetics,[6–8] self-renewal abilities,[9,10] and lineage output preferences after transplantation.[6–8,11,12] Further research has elucidated that HSC differentiation into various lineages is a continuous and intricate process, leading to the proposition of the "CLOUD HSPC" concept.[13] Consequently, analyzing the heterogeneity of HSC has important theoretical guiding significance and reference value for understanding the function of HSC, optimizing HSC transplantation, and elucidating the causes of hematological malignancies.

Although murine models have significantly advanced our knowledge of HSC biology, the insights gained by translating these studies into understanding of human reconstitution are limited due to substantial species differences.[14–17] Moreover, the complexity of polyclonal reconstitution in humans is greater than that of single-cell transplantation in mice, and the transplantation period is also much longer.[7,8] Given ethical considerations, studies on human HSC heterogeneity are mainly conducted by transplanting gene-edited HSC in the context of HSC genetic defects,[18–20] which cannot reflect the real state of hematopoietic reconstitution of normal human HSCs. Non-human primates

(NHPs), due to their high evolutionary and physiological similarity to humans and the consistency of their transplantation protocols with clinical protocols for human patients (both utilizing autologous mobilized CD34+ cell populations),[18,21,22] serve as an effective alternative for mimicking the reconstitution patterns and differentiation processes of human HSCs after autologous transplantation.

The current approach to track hematopoietic reconstitution after HSC transplantation in NHPs is to introduce viral vectors into HSCs and distinguish different HSC clones by integration site analysis (ISA). This approach has been utilized to reveal the diverse differentiation potentials and dynamic behaviors of HSC clones following autologous transplantation in NHPs. For instance, a clonal tracking study in Rhesus macaque by Kim et al confirmed the existence of 3 types of HSCs with different lineage output potential within CD34+ cells of mobilized peripheral blood.[21] Applying the mouse classification method,[8] the long-lived HSC clones in NHPs could be divided into myeloid-biased, lymphoid-biased, and balanced subtypes.[21] Moreover, such clones exhibiting lymphoid/myeloid bias might maintain for many years.[22] In addition, compared with mobilized peripheral blood, the transplantation reconstitution of bone marrow HSCs is closer to the hematopoiesis of NHPs in physiological environment. Most recently, Radtke et al[23] discovered that long-term persisting HSC clones in the bone marrow of Macaca nemestrina play an active role in the early recovery process of granulocytes and become the main resource of blood production as early as 50 days post-transplantation, rather than contributing to the blood system as previously reported.[24] However, the lineage-biased output behavior of HSC clones in bone marrow has not been further explored and elaborated.

Reconstitution of the blood lineages after autologous transplantation in NHPs occurs through the combined implantation, colonization, and differentiation of a large number of hematopoietic stem/progenitor cells. The lineage output behaviors of individual HSCs in the NHP autologous transplantation, especially for HSCs with multilineage differentiation, are not yet well clear. To better address this issue, we reanalyzed the public data of HSC clones after autologous bone marrow transplantation of NHPs and performed unbiased bioinformatic analysis of HSCs with long-term multilineage reconstitution. We found that there still existed a variety of single-lineage–biased output subtypes in the HSC population with multilineage differentiation, and different subtypes undergo transition to each other over time. This study expands the current knowledge of HSC heterogeneity, providing a reference for optimizing HSC transplantation strategies.

2. MATERIALS AND METHODS

2.1. Data selection and normalization

The ISA data used in this study were downloaded from https://github.com/KiemLab-RIS/ISA-Clone-tracking. The data at two time points for each of two NHPs (day 334 and 1265 for Z13624, and day 362 and 1309 for Z14004) were selected.[23] The contribution values of each clone into a given lineage at a given timepoint were calculated as read counts of this clone divided by all read counts in the given lineage and timepoint. Then, those 820 clones contributing to all five lineages (granulocyte, monocyte, B cell, T cell, and natural killer cell) were used for downstream analysis. To compare the five-lineage output characteristic across clones, we further transformed the contribution values of five lineages to have a sum of 1 for each clone.

2.2. Identification of HSC clonal subtypes

Hierarchical clustering was used to identify subtypes of HSC clones. Specifically, the matrix of output characteristic of all 820 clones was normalized to make margin sum of squares equal to one. Further, the normalized matrix was used to calculate the Euclidean distance matrix between clones which was used to perform hierarchical clustering. Finally, "cutree" function was used to divide the result of hierarchical clustering into six groups identifying as HSC clonal subtypes.

2.3. Quantification of lineage bias

We defined lineage bias score to quantify the output bias of HSC clonal subtypes. In detail, the output characteristic of all 820 clones was considered as the reference, and the distribution of contribution values was used as null distribution for each lineage. Then, for each lineage, the skewness of the distribution of contribution values in a given subtype compared with the null distribution, was calculated as lineage bias score. Specifically, the skewness for the proportion distribution between the given type and null distribution is calculated as follows.

$$\text{skew}\left(X_{i,j}\right) = E\left[\left(\frac{x_{i,j} - \mu_j}{\sigma_j}\right)^3\right]$$

Here, i means the given subtype and j means the given lineage. The final identification of lineage bias with the comprehensive assessment of the offset of the given subtype in other lineages and the offset of other subtypes in the given lineage. Roughly, the bias score (skewness) bigger than 2 is considered as the lineage biased.

2.4. Definition of α/β/γ HSC clonal subtypes

Four lineages (granulocyte, monocyte, B cell, and T cell) were used to define α/β/γ HSC clonal subtypes. For each clone, the FACS sorting ratio of the lineage was multiplied by the lineage contribution values for further calculation. According to the published study,[7,8] granulocytes plus monocytes (GM) compared to the B cells and T cells (B + T) (referred to as GM/(B + T)) contribution ratio bigger than 2 was defined as α-HSC clones, while the ratios between .25 and 2 were identified as β-HSC clones. The clones with the GM/(B + T) contribution ratio <.25 were classified into γ-HSC clones.

2.5. Statistics

Wilcoxon rank-sum test (two-sided) was used to detect the difference between two groups of samples.

3. RESULTS

3.1. Characterizing functional heterogeneity of HSCs in bone marrow of NHPs based on five-lineage output bias

Hematopoietic recovery within nine months after autologous transplantation in humans[18] and NHPs[23] is considered to be driven by short-term HSCs and a small number of long-term HSCs (LT-HSCs). After that, it tends to steady-state hematopoiesis, and LT-HSCs become the main source of blood lineages.[18,23] To explore the functional characteristics of HSCs that contribute to the five available lineages, we selected the data set of peripheral blood at two time points (11/12 and 42/43 months) after autologous transplantation of NHPs bone marrow CD34+ cells.[23] The dataset contains high-throughput DNA sequencing data of five blood cell lineage samples (granulocytes, monocytes, B cells, T cells, and natural killer cells). Cells with different integration site information can be distinguished by ISA, and the cells with the same integration site are considered to be from one clone. We included a total of 820 clones detected in all five lineages for further bioinformatic analysis (**Fig. 1A**).
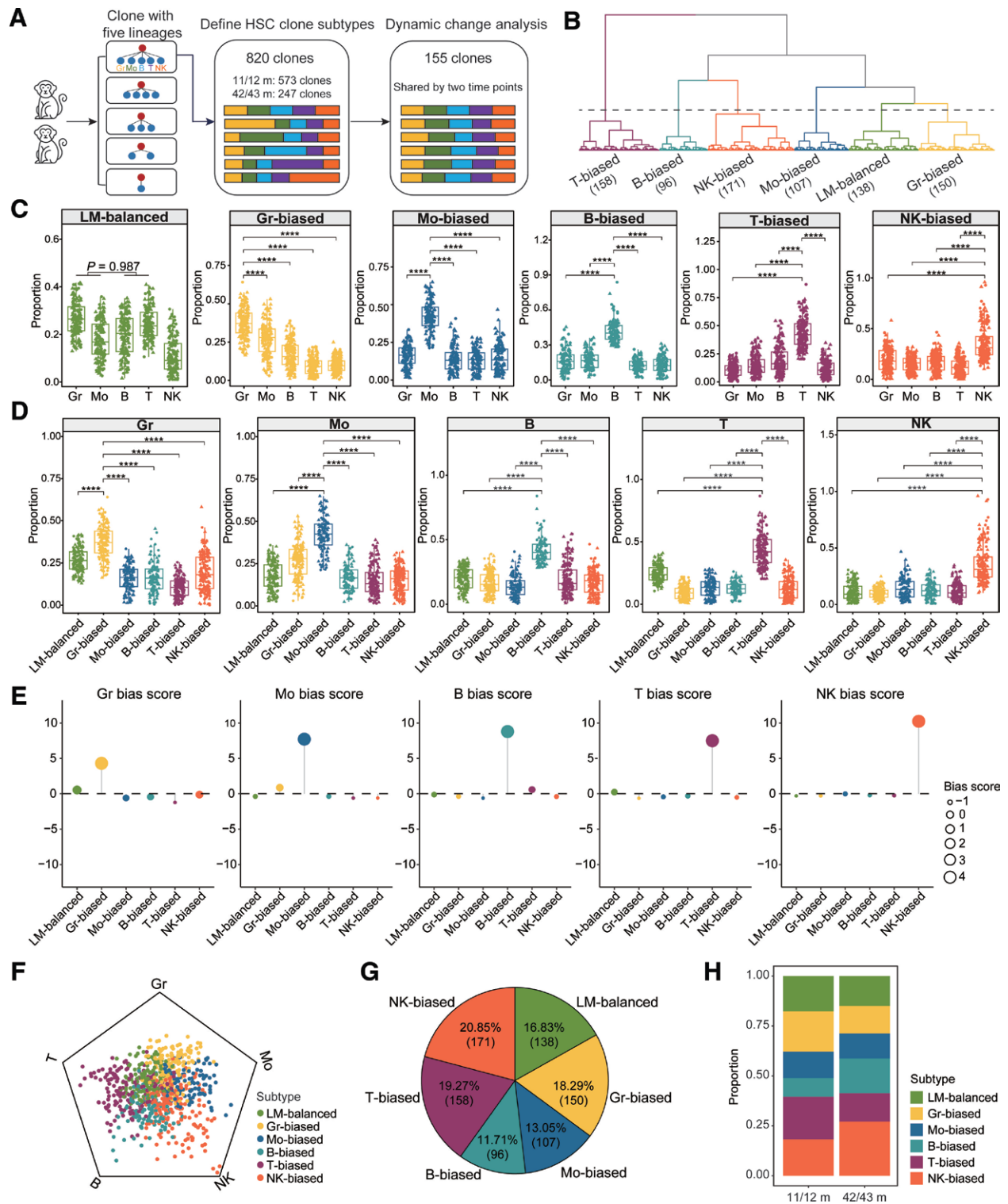
Figure 1. Systematic analysis based on five-lineage output bias reveals six subtypes of multipotent HSC clones in NHPs. (A) Workflow shows the screening of HSC clones with five-lineage output ability for analysis. (B) Hierarchical clustering shows six HSC clonal subtypes marked with different colors. The dotted line indicates the position to cut the tree for subtyping determination. The parentheses indicate the number of clones in each subtype. (C) Boxplot displays the proportion of the five lineages in each subtype. Wilcoxon rank-sum test is used to evaluate the significance of the difference and *P* value is labeled in the figure. Different samples are represented by different symbols (dots for Z13264 and triangles for Z14004). ****P* < .0001. (D) Box plot shows the proportion of each lineage in six subtypes. Wilcoxon rank-sum test is used to evaluate the significance of the difference and *P* value is labeled in the figure. Different samples are represented by different symbols (dots for Z13264 and triangles for Z14004). ****P* < .0001. (E) Lineage bias score indicates the bias degree of lineage output in each subtype compared to that of all clones. Both the length of the line and the size of the dot are proportional to the degree of bias. (F) Polygonal graph displays the distribution of clones in six subtypes. (G) Pie chart shows the proportion and numbers of clones in each subtype. (H) Bar plot displays the proportion of each subtype at two time points. B = B cell, B-biased = B cell-biased, Gr = granulocyte, Gr-biased = granulocyte-biased, HSC = hematopoietic stem cell, LM-balanced = lymphoid/myeloid balanced, m = month, Mo = monocyte, Mo-biased = monocyte-biased, NK = natural killer cell, NK-biased = natural killer cell–biased, T = T cell, T-biased = T cell-biased.
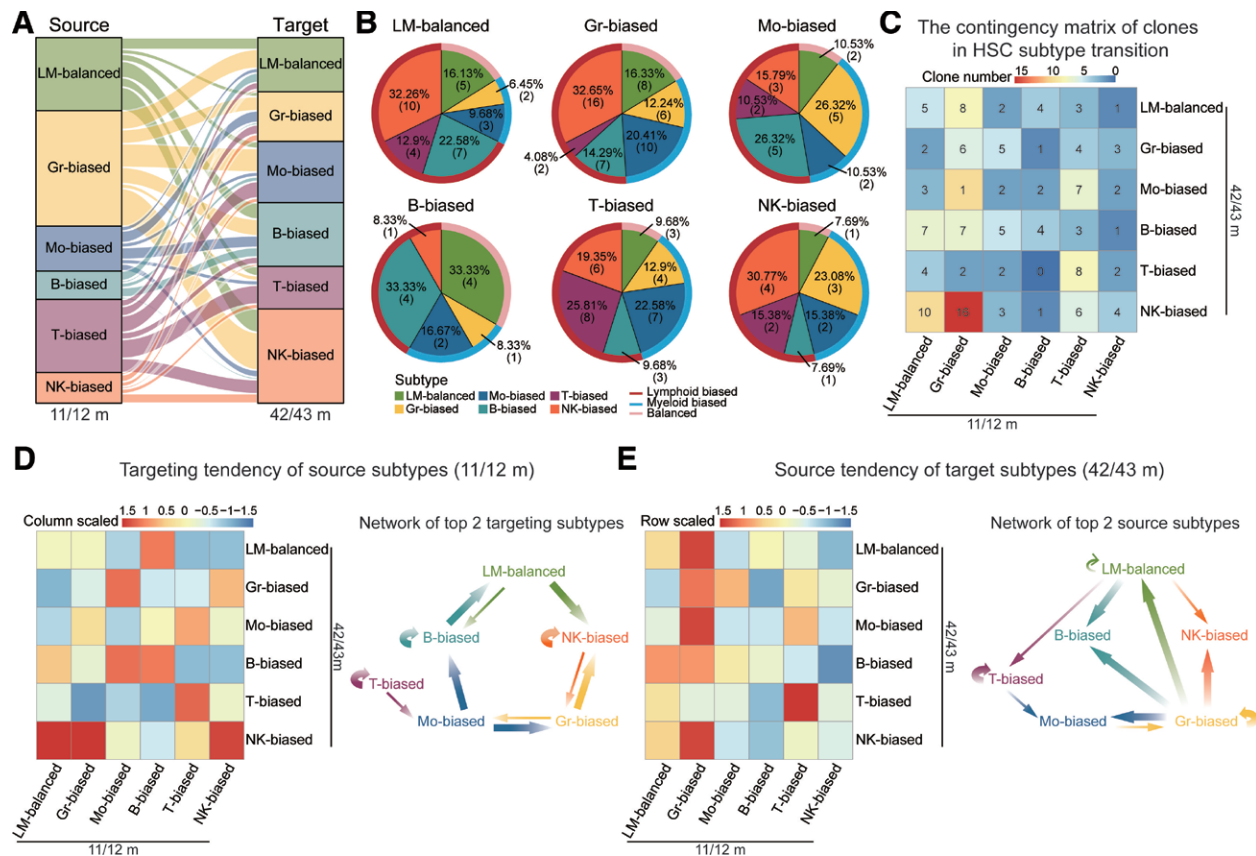
Figure 2. Dynamic changes in HSC clonal subtypes. (A) Sankey diagram displays the changes of each subtype over time. Clones shared by two time points were used. (B) Pie charts show the transition proportion of a specific subtype in the 11/12 months to either a different subtype or its own subtype in the 42/43 months. (C) Confusion matrix shows the assignment of the same clones to different subtypes at two time points. (D) Column scaled heatmap of (C) displaying the tendency of source subtypes (11/12 m) to be transitioned to target subtypes (42/43 m). Arrow network diagram illustrating the top 2 target subtypes for each source subtype. The tendency is calculated as the scaled value. Heavy arrows represent the first target subtypes. Thin arrows represent the second target subtypes. (E) Row scaled heatmap of (C) shows the tendency of targeted subtypes (42/43 m) coming from different source subtypes (11/12 m). Arrow network diagram illustrating the top 2 source subtypes for each target subtype. The tendency is calculated as the scaled value. Heavy arrows represent the first source subtypes. Thin arrows represent the second source subtypes. B = B cell, B-biased = B cell-biased, Gr = granulocyte, Gr-biased = granulocyte-biased, HSC = hematopoietic stem cell, LM-balanced = lymphoid/myeloid balanced, m = month, Mo = monocyte, Mo-biased = monocyte-biased, NK = natural killer cell, NK-biased = natural killer cell–biased, T = T cell, T-biased = T cell-biased.

To better distinguish the lineage output characteristics of clones, we comprehensively selected six subtypes for interpretation based on computational evaluation, such as elbow methods, and the lineage output performance of subtypes (**Fig. 1B**). Through comparison analyses of the proportion of five lineages in each subtype (**Fig. 1C**) and the proportion of each lineage across different subtypes (**Fig. 1D**), we found that the five-lineage output of each subtype showed unique characteristics. The one subtype exhibited balanced output of lymphoid and myeloid lineages ($P = .987$), thus defined as lymphoid/myeloid balanced (LM-balanced). The other five subtypes each had a high output bias of a single lineage, which was in concert with the results of the cell lineage bias score (**Fig. 1E**), thus named as granulocyte-biased (Gr-biased), monocyte-biased (Mo-biased), B cell-biased (B-biased), T cell-biased (T-biased), and natural killer cell–biased (NK-biased). These six subtypes were projected into the polygonal graph, and the results showed that the five single-lineage–biased subtypes were distributed in the corresponding lineage dimension, and LM-balanced was distributed in the center but biased toward granulocytes and T cells (**Fig. 1F**). In fact, compared to other lineages, the proportion of granulocytes and T cells in LM-balanced was relatively higher (**Fig. 1C, D**).

We found that the proportion of different subtypes in all clones was balanced (**Fig. 1G**), and all six subtypes were present at different transplantation time points, but the proportion of

each subtype was different (**Fig. 1H**). After more than 40 months of transplantation (42/43 m), the proportion of B-biased and NK-biased increased, the proportion of Gr-biased and T-biased slightly decreased, and the proportion of LM-balanced and Mo-biased changed little (**Fig. 1H**). These results revealed significant functional heterogeneity of long-term multipotent HSCs in bone marrow of NHPs. Although these HSC clones all had the ability to produce five lineages, they also showed an output preference for a single lineage. This finding further emphasized the complexity and diversity of HSCs in maintaining the balance of the blood system.

3.2. Dynamic changes in lineage output bias of HSC clones over time

We found that the proportion of different subtypes changed with the increase in transplantation time, suggesting that the output bias of HSC was not static (**Fig. 1H**). To investigate this issue, we used a total of 155 clones that could be detected at both time points (11/12 and 42/43 m) for further analysis (**Fig. 1A**). Consistent with previous data (**Fig. 1H**), the proportion of NK-biased ($P = .0001$) and B-biased ($P = .0165$) increased significantly, and the proportion of Gr-biased decreased significantly ($P = .0002$). Further, we tracked the changes in these clones after transplantation for more than 40

months, and the results showed that the types of these subtypes changed. We observed that all subtypes except B-biased showed the ability to transition to all subtypes (**Fig. 2A**). Specifically, the six subtypes of lymphoid biased showed a strong ability to maintain self-type compared with other subtypes. Most of the clones of these six subtypes (B-biased, 33.33%; NK-biased, 30.77%; T-biased, 25.81%) retained their own lineage output characteristics, while the myeloid-biased subtypes (Gr-biased, 12.24%; Mo-biased, 10.53%) showed a relatively weak ability to maintain self-type. In addition, we also found that compared with the transition to the myeloid-biased subtype, each subtype was more inclined to transition to the lymphoid-biased subtypes (**Fig. 2B**). Notably, we also observed that other subtypes rarely transitioned to T-biased (**Fig. 2C, D**), which is consistent with the results of hierarchical clustering where T-biased and the other five subtypes belong to two different branches (**Fig. 1B**). This implies that the chances of other types turning into T-biased are relatively low. Particularly, after transplantation for more than 40 months, except for T-biased, most of the clones of other subtypes were contributed by Gr-biased (**Fig. 2C, E**), while few clones of Gr-biased were derived from other subtypes, leading to a decreased proportion of Gr-biased in the later transplantation period (**Fig. 2A**). T-biased were mainly composed of self-type clones after more than 40 months post-transplantation (**Fig. 2C, E**). In summary, we reveal the variability of the lineage output of HSCs, and the lineage output bias will change with the increase in transplantation time.

3.3. Five-lineage classification refined the traditional lymphoid/myeloid bias classification

Eaves and colleagues used single-cell transplantation to reveal that adult mouse bone marrow HSCs exhibited lineage output bias.[7,8] According to the flow ratio of GM/(B + T) detected in peripheral blood at 16 weeks after transplantation, mouse bone marrow HSCs were divided into myeloid-biased HSCs (α-HSCs), balanced HSCs (β-HSCs), and lymphoid-biased HSCs (γ/δ-HSCs). And this heterogeneity could be traced back to the pre-HSCs (pre-HSCs) and nascent HSCs during embryonic development.[25] We adopted the same strategy to classify HSCs in bone marrow of NHPs, and three subtypes of α-, β-, and γ-HSCs were detected in different individuals (**Fig. 3A, B**). Consistent with mice, the proportion of β-HSCs was the highest (62.93%), the proportion of γ-HSCs was the lowest (3.42%), and there was repeatability between different individuals (**Fig. 3A, B**). The three subtypes were projected into the polygonal graph, and the results showed that α-HSCs were distributed toward the directions of granulocytes and monocytes, γ-HSCs were distributed toward the directions of B cells and T cells, and β-HSCs were mostly gathered in the middle, which also matched the lymphoid/myeloid bias characteristics of the three HSC subtypes (**Fig. 3C**). The changes of different subtypes with the increase of transplantation time were further tracked. After more than 40 months post-transplantation, all the three subtypes still existed, and the proportion of β-HSCs remained the highest among the three subtypes (**Fig. 3D**), which was consistent with the highest proportion of balanced subtype in G-CSF mobilized peripheral blood of NHPs.[21] We further analyzed the dynamic changes of different subtypes at two time points. Although the proportion of different subtypes did not change much (**Fig. 2E**), there was a mutual transition among different subtypes. Specifically, most of the β-HSCs maintained their own subtype, and a small proportion of clones transitioned to α-HSCs. The ability of α-HSC to maintain its own subtype was weak, most clones transitioned to β-HSCs and a few clones transitioned to γ-HSCs (**Fig. 3F**). These results revealed that the subtypes of the lymphoid/myeloid bias classification also showed changes in lineage output with the transplantation time.

To investigate the relationship between the six subtypes defined by the five-lineage classification and the traditional α-, β-, and γ-HSCs, we analyzed the proportion and clone numbers of the six subtypes contained in α-, β-, and γ-HSC subtypes (**Fig. 3G**). The results showed that α-HSCs mainly contained Gr-biased and Mo-biased, but no B-biased and T-biased, γ-HSCs only contained lymphoid-biased clones, most of which were B-biased and T-biased, while the vast majority of LM-balanced were of the β-HSCs, suggesting the roughly consistency of different classification methods. Interestingly, the β-HSCs also contained a considerable amount of B-biased and T-biased, which might have been related to the low standard threshold setting of the β definition. The five-lineage classification method was able to further refine clones with a single lineage bias based on the traditional lymphoid/myeloid bias classification. It is worth noting that the traditional lymphoid/myeloid bias classification method does not consider NK cells. Through the joint analysis of the two classification methods, we found that NK-biased was distributed in all three subtypes, but it was more concentrated in β-HSCs and α-HSCs (**Fig. 3G**). Further, we found that after a prolonged period of transplantation, the proportion of the six subtypes in α-, β-, and γ-HSCs changed, but the distribution was consistent with the results of 11/12 months post-transplantation. This showed that although there was a more complex subtype transition within the six subtypes (**Fig. 2A**), the subtype composition reflected in α-, β-, and γ-HSCs was stable (**Fig. 3H**). In general, by using two different classification strategies, we revealed that HSCs with long-term multilineage reconstitution ability in bone marrow of NHPs showed highly heterogeneous lineage differentiation behavior. These HSCs could undergo mutual transitions between different types but generally maintained steady-state hematopoiesis.

4. DISCUSSION

The lineage output heterogeneity of HSCs in bone marrow of NHPs is currently unclear. We focused on the HSC clones with five-lineage totipotent output after 10 months of autologous transplantation in NHPs. Through unbiased bioinformatics analysis, we found that such five-lineage totipotent output HSCs still exhibited significant lineage bias, including one subtype of LM-balanced output and five subtypes of single-lineage–biased output. Compared with the traditional lymphoid/myeloid bias classification,[7,8,25] the five-lineage classification method incorporated more comprehensive information, and allowed us to identify more detailed lineage biases, reflecting the heterogeneity of lineage output of HSCs in NHPs. Nevertheless, accumulating findings suggest the greater heterogeneity of HSCs, for instance, megakaryocyte-biased HSCs have been found in the mouse bone marrow.[26] These results indicated that introducing complete lineage information, such as the further addition of erythrocyte and megakaryocyte lineages, may comprehensively enhance our knowledge of HSC heterogeneity.

The traditional method of defining HSC subtypes is to classify HSC at a single static time point after transplantation,[7,8] which ignores the fact that HSCs may exhibit different lineage differentiation bias and functional characteristics at different developmental stages or under different physiological states. This heterogeneity may be affected by cell-intrinsic factors (such as gene expression, epigenetic modification) and cell-extrinsic factors (such as microenvironment, signaling pathways).[27] Uncovering the heterogeneity of HSCs through dynamic tracking, which reveals that the lineage output bias of HSCs changes over time, poses a challenge to HSC heterogeneity studies based on a single time point. By tracking and comparing clones at different time points, we found that different subtypes underwent significant mutual transitions over time. Notably, each of six subtypes was more likely to transition to lymphoid lineage–biased than myeloid lineage–biased subtypes.
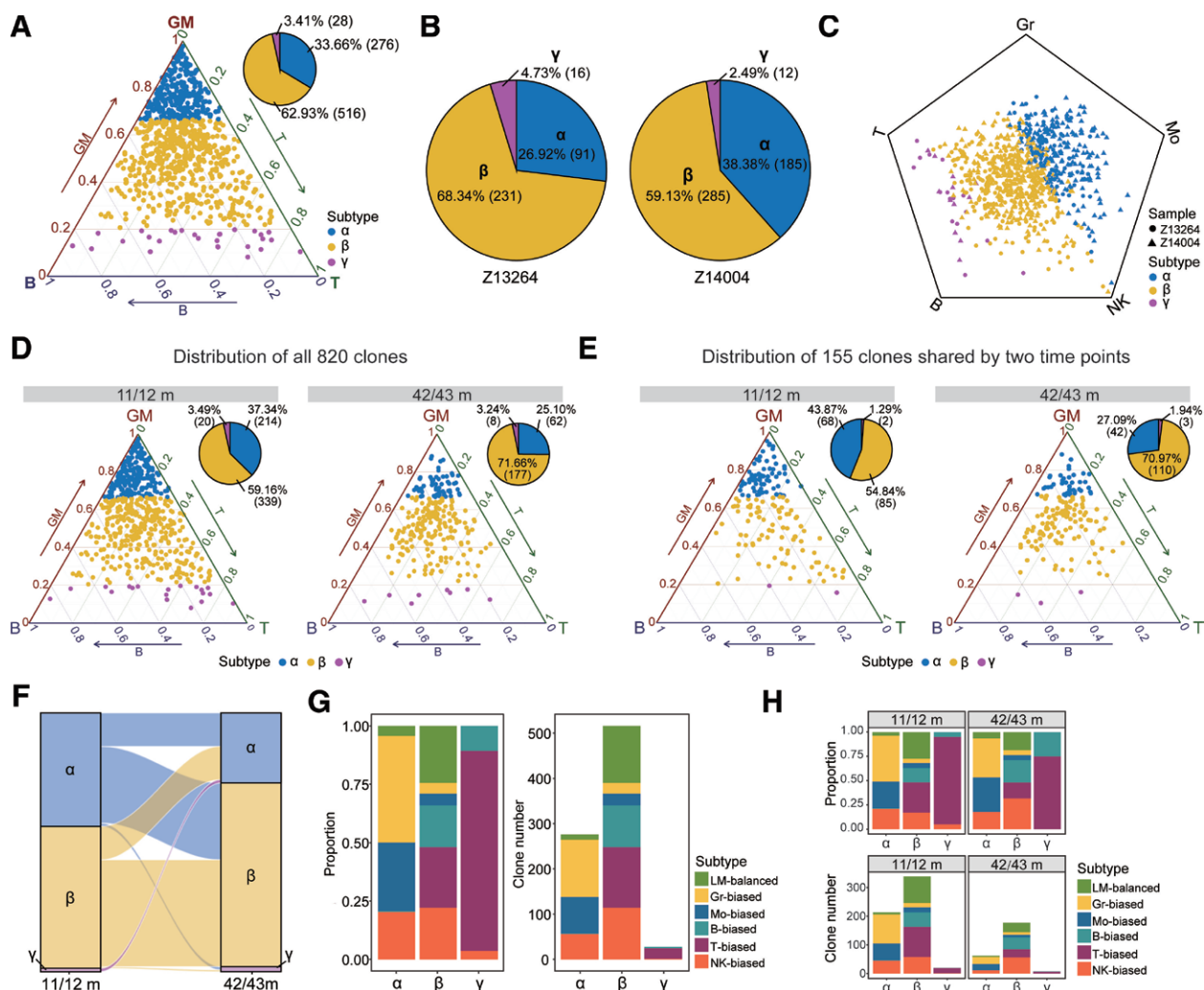
Figure 3. Comparative analysis between the five-lineage classification and the lymphoid/myeloid bias classification. (A) A ternary plot shows the distribution of HSC clones assigned into three subtypes defined based on their lymphoid/myeloid output bias. α = myeloid-biased; β = balanced; γ = lymphoid-biased. Pie chart displays the proportion and clone numbers of each subtype. (B) Pie charts display the proportion and clone numbers of each subtype in each sample for Z13264 and Z14004, respectively. (C) Polygonal graph displays the distribution of clones in the three subtypes. (D and E) The ternary plots and pie charts show the distribution and proportion of the three subtypes at two time points. (D) is for all 820 clones, but specific to one of the two time points. (E) is for the 155 clones shared at two time points, but specific to two of the two time points. (F) Sankey diagram shows the transition of subtypes. Only 155 clones shared by two time points were used. (G and H) The relationship between the subtypes defined by the five-lineage classification and the traditional classification by lymphoid/myeloid bias. The proportion (G, left panel; H, top panel) and clone numbers (G, right panel; H, bottom panel) of the six subtypes in α-, β-, and γ-HSCs. (G) is for all clones at two time points. (H) is specific to one of the two time points. B = B cell, B-biased = B cell-biased, Gr = granulocyte, Gr-biased = granulocyte-biased, HSC = hematopoietic stem cell, LM-balanced = lymphoid/myeloid balanced, Mo = monocyte, Mo-biased = monocyte-biased, NK = natural killer cell, NK-biased = natural killer cell–biased, T = T cell, T-biased = T cell-biased.

Considering that both NHP transplants were under three years of age, and post-transplantation ranged from 11/12 to 42/43 months, the NHP is in the development and sexual maturity stage. The bone marrow microenvironmental niche of NHPs may still be undergoing dynamic development. In addition, increasing evidence suggests that sex hormones regulate HSC self-renewal, differentiation, and proliferation during mouse puberty.[28,29] Clones of different subtypes may undergo changes in lineage output in order to adapt to this growth and development process. Meanwhile, these changes still maintain hematopoietic balance. However, it remains to be further investigated whether the dynamic changes of different subtypes are regulated by cell-extrinsic factors, and/or derived from cell-intrinsic factors such as transcriptional lineage-priming programs and metabolic heterogeneity.[27,30] These results suggest that by monitoring the lineage output of individual HSC clones at various time points, we can comprehensively understand the functional heterogeneity of HSCs in vivo.

In this study, we only selected clones with five lineages detected at the same time point as multipotent HSC clones. This selection strategy may underestimate the number of reconstitutions of HSC, because some five-lineage reconstituted HSC clones may be excluded given the lack of one or more lineages due to limitations (eg, drop-out events by limited sampling rate or detection sensitivity) in ISA.[31] Better clonal tracing techniques will help address these limitations. Nevertheless, the current number of clones is sufficient to allow us to observe the reproducible results among different individuals, providing valuable information.

In summary, these results enrich our understanding of the heterogeneity of HSCs in NHPs and provide important insights into human research.

AUTHOR CONTRIBUTIONS

B.L., Y.L., Y.N., and Z.L. designed the research; M.Z., D.L., and Z.L. collected, analyzed, and interpreted data; M.Z., D.L., Z.L. wrote the manuscript; and all the authors read, revised, and approved the final manuscript.

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