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# The Road Map for Megakaryopoietic Lineage from Hematopoietic Stem/Progenitor Cells

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## SUMMARY

Megakaryocytes (Mgks) are terminally differentiated blood cells specified to produce platelets, whereas hematopoietic stem cells (HSCs) are the most undifferentiated blood cells that retain multipotency to produce all kinds of blood cells. As such, these two cell types reside at the bottom and the top of the hematopoietic hierarchy, respectively. In spite of this distance, they share several important cell surface molecules as well as transcription factors.

In the conventional step-wise differentiation model, HSCs gradually lose their self-renewal capacity and differentiate into multipotent progenitors (MPPs), which is the first branch point of myeloid and lymphoid lineage. In this model, common myeloid progenitors can differentiate into bipotent Mlgk/erythroid progenitors (MEPs), and MEPs eventually differentiate into unipotent mature Mgks. However, it has been recently reported that a subpopulation within the HSC and MPP compartments demonstrates an Mlgk-biased differentiation potential. These reports imply that revisions to the HSC-to-Mlgk differentiation pathway should be discussed. In this review, we summarize recent findings about Mlgk differentiation from HSCs and discuss future directions in this research field. *STEM CELLS TRANSLATIONAL MEDICINE* 2017;6:1661–1665

## SIGNIFICANCE STATEMENT

Transplant experimental models and single-cell analysis data reveal that the hematopoietic stem cell (HSC) compartment contains a subpopulation with megakaryocyte (Mlgk) lineage-biased potential. Mlgk-biased progenitor cells also can be detected in the multipotent progenitor fraction, suggesting that Mgks could emerge through a non-step-wise differentiation pathway from HSCs, especially in stress-induced hematopoiesis.

## INTRODUCTION

The hematopoietic system constantly generates a precise number of blood cells with diverse functions. These functions are maintained by hematopoietic stem cells (HSCs), characterized by self-renewal capacity and multipotent differentiation potential. This rare subpopulation of blood cells provides a huge number of peripheral blood cells throughout an individual's lifetime [1].

Platelets are small, anucleated fragments of blood cells that are generated from polyploid megakaryocytes (Mgks) and play a critical role in homeostasis [2]. In the classical “step-wise” hierarchical hematopoiesis model, HSCs produce committed progenitors with decreasing self-renewal capacity and restricted lineage differentiation potential. The bifurcation of myeloid/lymphoid lineages first occurs within multipotent progenitors (MPPs) during differentiation. The myeloid lineage progenitors further lose differentiation potential into granulocyte/macrophage and erythroid lineages and eventually produce unipotent Mlgk progenitors (MKPs) [3]. This differentiation model simplifies the complexity of the hematopoietic system and has been widely accepted over the past decades. However, recent studies with novel prospective

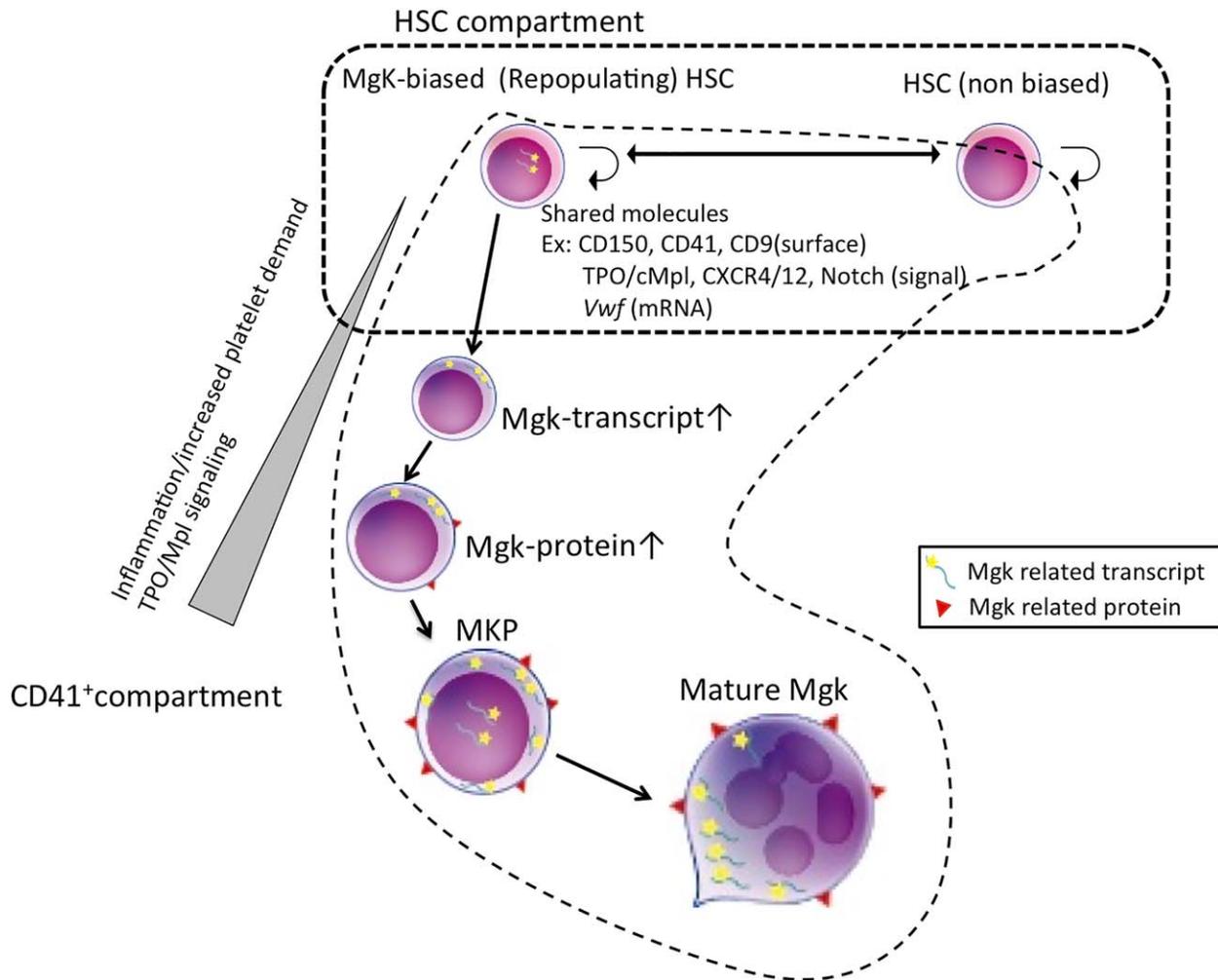
isolation techniques and single-cell functional/molecular analyses of HSCs and Mgks have revealed that these two cell types share a remarkable number of surface molecules, transcription factors, and cytokine signaling pathways. This suggests that Mlgk might be directly differentiated from HSC, possibly bypassing the Mlgk/erythroid progenitor (MEP) stage [4]. In this paper, we summarize the recent findings within the topic of megakaryopoiesis and propose a revised road map for Mlgk lineage differentiation.

## SHARED SURFACE RECEPTORS, SIGNALING, AND GENE EXPRESSION PROFILES IN HEMATOPOIETIC STEM CELLS AND COMMITTED Mlgk LINEAGE CELLS

HSCs and Mgks share many common features, as previously described (Fig. 1) [4]. As an example, the thrombopoietin (TPO) signaling is one of the most essential cytokine signaling pathways in both HSCs and Mgks. TPO was originally identified as a critical growth factor for platelet production [5]. However, studies using *Mpl*<sup>-/-</sup> mice demonstrated that *Mpl*, which is a TPO receptor, also plays an essential role for maintenance of HSCs [6]. HSCs derived

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**Figure 1.** Shared surface receptors and signaling in HSCs, Mgk-biased HSCs, and committed Mgks. In the immunophenotypic HSC compartment (mainly the CD41<sup>+</sup> subpopulation), Mgk-biased HSCs exist. HSCs, Mgk-biased HSCs, and MKPs share several surface molecules. Transcripts of Mgk-related genes, such as *Vwf* or *CD42b*, can be detected in Mgk-biased HSCs, but protein synthesis is inhibited in the steady state. External signaling such as inflammatory cytokine or TPO/Mpl signaling can trigger protein synthesis, induce Mgk lineage differentiation, and generate adequate numbers of platelets during emergency thrombocytosis. Abbreviations: HSC, hematopoietic stem cell; Mgk, megakaryocyte; MKP, Mgk progenitor; TPO, thrombopoietin.

from *Mpl*<sup>-/-</sup> mice showed a significantly reduced long-term repopulating capacity, suggesting that TPO/Mpl signaling is functionally essential in HSC activity [6]. Another study demonstrated an indispensable role of TPO/Mpl signaling in HSC quiescence [7]. In humans, congenital defects or a loss-of-function mutation of *Mpl* causes severe thrombocytopenia, called congenital amegakaryocytic thrombocytopenia (CAMT). Patients with CAMT have a high risk of bone marrow failure, suggesting that TPO/Mpl signaling is also important for maintenance of human HSCs [4]. These data imply that TPO/Mpl signaling might play a critical bridging role between HSCs and Mgks. In addition, other shared signaling pathways between HSCs and Mgks, including CXCR4/CXCL12 [8], [9], Notch [10, 11], and Stem cell factor/c-Kit signaling [12], have also been reported [4].

Furthermore, various surface molecules are co-expressed on HSCs/MPPs and Mgks. Indeed, the majority of surface markers so far used for isolation of MKPs, such as CD150 [13], CD41 [14], and CD9 [15], are also expressed in the entire population of, or a fraction of, HSCs. CD150, known as SLAMF1, is widely used for

purification of mouse HSCs [13] and is also expressed on Mgk lineage cells, including MKPs and platelets [16]. CD41 (integrin  $\alpha$ IIb) non-covalently associates with CD61 (integrin  $\beta$ 3) to form the integrin  $\alpha$ IIb $\beta$ 3 complex on Mgks and platelets [4], which plays a critical role in thrombus formation [17]. Additionally, CD41 is expressed on the surface of a subpopulation of long-term HSCs (LT-HSCs) and MPPs in mice. CD41 expression in Lineage Sca1<sup>+</sup>/c-Kit<sup>+</sup> mouse hematopoietic stem/progenitor fraction (LSK) increases with aging and is related to the myeloid differentiation potential of HSCs [14, 18]. From the similar expression pattern of these molecules arises the possibility that Mgks may directly differentiate from HSC.

In a closely associated context, a subpopulation of HSCs was reported to express mRNA of von Willebrand factor (vWF) [19], which is mainly found on the surface of injured endothelial cells, mature Mgks, and platelets. On the other hand, CD42b, which is the vWF receptor, was believed to be exclusively expressed on the surface of Mgks and platelets [16]. Unlike CD41, immature progenitor cells such as MPPs and LT-HSCs do not express CD42b in

steady-state conditions [16]. Despite such a classical view, we demonstrated CD42b surface expression in a CD34<sup>-</sup>/CD41<sup>+</sup>/CD150<sup>+</sup> LSK population, a subset of HSCs, at the hematopoietic recovery phase after 5-fluorouracil treatment, prompting further investigation [16]. Indeed, single-cell gene expression analysis revealed that a subpopulation of CD41<sup>+</sup> LSK expresses nuclear *CD42b* mRNA even in the steady state [16], although it was not clear whether nuclear *CD42b* mRNA-positive CD41<sup>+</sup> LSKs were functional LT-HSCs. Furthermore, recent improvement of single-cell RNA sequencing techniques enables comprehensive gene expression analyses at single-cell resolution [20]. Whole transcriptome analysis using single purified HSCs demonstrated that a subpopulation of HSC compartment expresses Mlgk-specific genes [21].

Another group demonstrated the regulatory mechanism of the expression of Mlgk-related genes in HSCs [22]. They showed that the phenotypic HSC compartment contains stem-like Mlgk lineage-committed progenitors (SL-MKPs), which share similar characteristics with HSCs but retain platelet-restricted differentiation capacity. SL-MKPs reside in the CD41<sup>+</sup> subpopulation of phenotypic HSC and express Mlgk-specific mRNAs while translation of these mRNAs into protein is suppressed in SL-MKPs in the steady state. Inflammation signals, such as activation of the interferon signaling pathway, trigger Mlgk differentiation and platelet production through Mlgk-specific mRNA translation in SL-MKPs. The authors of this paper conclude that cell-cycle regulators such as p27 and p57 under strict control of FoxO3a suppress translation of such Mlgk-specific mRNAs in the steady state [22]. Although further investigation is required, transcriptional/translational regulation of Mlgk-specific genes in the HSC compartment may be a key to understand underlying mechanisms in the commitment to Mlgk lineage from HSCs.

#### Mlgk LINEAGE COMMITMENT WITHIN THE HSC COMPARTMENT

Heavily shared molecular markers between HSCs and Mlgks have raised the possibility that Mlgk lineage specification could occur at a more immature stage than that postulated in the classical step-wise differentiation model [4]. In the adult mouse hematopoietic system, HSCs are significantly enriched in either CD34<sup>low</sup>LSK [1] or CD150<sup>+</sup>/CD48<sup>-</sup> LSK cells [13]. In the classical differentiation model, the most immature myeloid progenitors, defined as common myeloid progenitors (CMPs), are derived from MPP and differentiate into bipotent granulocyte/macrophage progenitors and MEPs, the latter of which eventually differentiate into MKPs while losing their capacity to differentiate into the erythroid lineage (Fig. 2A) [3]. However, it was recently reported that a phenotypic CMP population is highly heterogeneous when analyzed by single-cell RNA sequencing, while Mlgk-specific genes, particularly transcription factors, are detected in a subpopulation of CMPs, but not in MEPs [23]. Indeed, we demonstrated that a CD42b-positive subpopulation of CMPs represents unipotent MKPs [16]. CMPs could be a mixture of heterogeneous progenitors such as unipotent myeloid and Mlgk progenitors, which may support the concept that Mlgk lineage commitment occurs at stages close to HSCs.

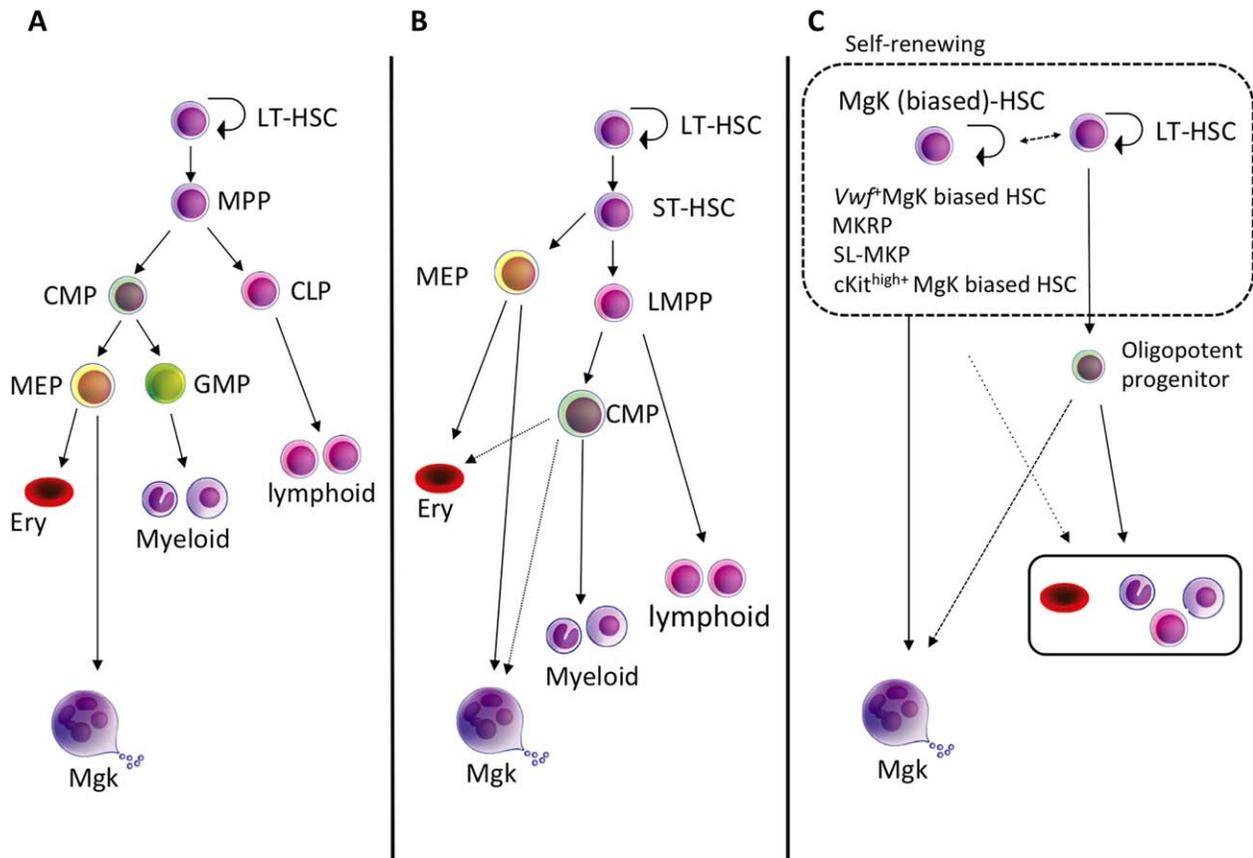
There are multiple lines of evidence showing that the megakaryopoietic pathway is bifurcated earlier than assumed in the classical model (Fig. 2B) [10, 19, 24, 25]. Flt3<sup>+</sup> LSKs are characterized as lymphoid-primed multipotent progenitors (LMPPs), which notably lose Mlgk/erythroid differentiation potential, retaining

granulocyte/macrophage potential [24]. While Mlgk/erythroid differentiation potential from LMPPs has been shown before, the actual capacity of LMPPs to produce Mlgk is reportedly significantly lower than that of HSCs or MPPs [18, 26, 27]. Additionally, it was reported that Notch signaling plays an important role in Mlgk lineage specification from HSCs in an in vitro co-culture system [10]. In this report, the authors suggested that Notch signaling promotes the alternative Mlgk differentiation pathway from HSCs, which could bypass the MEP stage.

More recently, several studies using novel single-cell functional analysis methods suggested that mouse LT-HSC populations are functionally heterogeneous and the commitment into Mlgk lineage is already made within a subpopulation of the LT-HSC compartment (Fig. 2C) [25]. Indeed, 10%–15% of the highly purified LT-HSC population can directly differentiate into Mlgk without cell division in single-cell in vitro culture. The same population also includes highly proliferating cells producing 100–1,000 single-lineage Mlgks [16, 25, 28]. Furthermore, the paired daughter assay shows Mlgks can be differentiated from HSCs even at the first cell division [25]. Although in vitro assays do not always perfectly simulate in vivo hematopoiesis, these data do support the possibility that the subpopulation of HSCs possesses Mlgk-biased/restricted capacity, and Mlgks may directly differentiate from them.

Data from a single-cell transplant report demonstrated that purified HSC populations (CD34 LSK) contain several lineages of biased HSCs, such as myeloid-biased, lymphoid-biased, and Mlgk/erythroid-biased HSCs [25]. These data implied that fate decision to a specific lineage from multipotent HSCs might be made at the LT-HSC level and also suggested that conventional, step-wise, hierarchical, hematopoietic differentiation models might need to be revised. They also demonstrated that the CD150<sup>+</sup>/CD41<sup>+</sup> subpopulation of a mouse LT-HSC population (CD150<sup>+</sup>/CD41<sup>+</sup>/CD34<sup>-</sup> LSK) contains Mlgk-restricted repopulating cells (MKRPs), which showed restricted Mlgk/platelet differentiation capacity and self-renewal capacity in vivo. SL-MKPs (as discussed previously) also reside as a subpopulation of the CD41<sup>+</sup>LT-HSC population (CD41<sup>med</sup>/CD150<sup>+</sup>/CD48<sup>-</sup>/c-Kit<sup>+</sup>/Lin<sup>-</sup>) [22]. Indeed, our single-cell culture data revealed that the frequency of pure Mlgk colonies from the CD150<sup>+</sup>/CD41<sup>+</sup> LSK population was much higher than that of the CD41<sup>+</sup> LSK population [16]. We also demonstrated that continuous TPO/Mpl signaling was key for Mlgk differentiation from HSCs, bypassing bipotent MEPs [16]. Additional clarity comes from studies (using *Vwf* as a genetic marker) that showed a subpopulation of *Vwf*-positive HSCs that possesses Mlgk-biased differentiation capacity and resides at the apex of the HSC hierarchy; these cells expand within the HSC compartment during aging [19, 21]. These data also supported the concept that the fate decision of the Mlgk lineage is made at the LT-HSC level. Our group also demonstrated that the CD150<sup>+</sup>/CD41<sup>+</sup> LSK population expresses *Vwf* at the single-cell level, and the CD41<sup>+</sup> LSK subpopulation showed exclusively high Mlgk differentiation capacity [16].

Shin and colleagues reported that c-Kit<sup>high+</sup> HSC (CD150<sup>+</sup>/CD34 LSK) showed impaired self-renewal activity and megakaryocytic bias [29]. This population produces Mlgks and platelets more rapidly in vitro and in vivo, and c-Kit<sup>low</sup> HSCs were shown to be more primitive and quiescent than c-Kit<sup>high+</sup> HSCs, implying that the Mlgk-biased subpopulation of HSCs was not at the apex of the hematopoietic hierarchy [29]. To clarify, if two distinct subsets of Mlgk-biased HSCs exist at the apex and the sub-apex of the hematopoietic system, further investigation is required. In actuality, these concepts were mainly based on data from transplant/



**Figure 2.** The models of Mgk differentiation from HSCs. **(A):** The classical “step-wise” hierarchical hematopoiesis model. The bifurcation of myeloid/lymphoid lineage first occurs in MPPs, and MKPs eventually generate from MEPs as the progeny of CMPs. **(B):** Alternative model based on the identification of LMPPs. The bifurcation of Mgk lineage first occurs during differentiation from HSCs. LMPPs lose almost all Mgk/erythroid differentiation potential. **(C):** Proposed model from recent reports. An immunophenotypic-defined HSC population contains a functionally heterogeneous, Mgk-biased/restricted subpopulation of HSCs that directly gives rise to MKPs and bypasses the MEP stage. Abbreviations: CLP, common lymphoid progenitor; CMP, common myeloid progenitor; Ery, erythroid; HSC, hematopoietic stem cells; LMPP, lympho-myeloid primed progenitor; LT-HSC, long-term hematopoietic stem cells; MEP, Mgk/erythroid progenitor; MgK, megakaryocytes; MKRP, Mgk-repopulating progenitors; MPP, multipotent progenitor; SL-MKP, stem-like Mgk committed progenitor; ST-HSC, short term HSC.

culture using a purified subpopulation of HSCs, which only reflected the highly stressed hematopoietic condition.

According to the results from a *Flt3* lineage tracing a mouse model that genetically marks the progeny of *Flt3*-expressing cells, all lineages (including platelets and erythrocytes) can be produced from *Flt3*<sup>+</sup> hematopoietic progenitors like the LMPP fraction *in vivo* [30]. Although the possibility of transient *flt3* promoter activation at the HSC level might be a concern, the fate decision mechanism that specifies lineage from HSCs in the steady state might be different from stress conditions such as hematopoietic reconstitution after transplant or chemotherapy. Further analyses would be needed for understanding platelet generation from steady-state HSCs.

#### MEGAKARYOCYTOPOIESIS IN THE HUMAN HEMATOPOIETIC SYSTEM

The classical, stepwise hematopoietic differentiation model has been widely accepted to be the same in humans as in mice [31]. However, an alternative differentiation model was also suggested

in the human hematopoietic system, which would mirror the mouse system [32, 33]. Using human cord blood and bone marrow cells, single-cell analysis similarly revealed that the Mgk lineage fate decision was made within the CD34<sup>+</sup>/CD38<sup>+</sup> HSC-enriched compartment and that the CD34<sup>+</sup>/CD38<sup>+</sup> CMP/MEP populations are both heterogeneous [34], retaining less differentiation capacity into Mgks [32]. They also demonstrated that Mgks are generated from intermediate oligopotential progenitors in human fetal hematopoiesis, suggesting that the Mgk lineage differentiation systems in fetal liver and adult bone marrow are distinct from each other [32]. Further studies are required to prove the existence of Mgk-biased HSCs or progenitor cells in the human hematopoietic system, given that *in vivo* functional analysis at the single-cell level is much more difficult in humans than in mice [35].

#### CONCLUSION

Several recent studies demonstrated that the commitment to the Mgk lineage is decided at a much earlier stage than previously expected. The existence of lineage-biased HSCs, including Mgk-

biased/restricted HSCs, is also demonstrated. Although the physiological roles of this unique class of cells in hematopoiesis and hematologic diseases are still not fully elucidated (especially in the human hematopoietic system), these findings provide new insights into the hematopoietic regulatory mechanism.

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#### AUTHOR CONTRIBUTIONS

H.N., K.N., and S.C.: manuscript writing.

#### DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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