


CONCISE REVIEW

The heterogeneity of megakaryocytes and platelets and implications for ex vivo platelet generation

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Abstract

Platelets, the chief effector of hemostasis, are small anucleate blood cells generated from megakaryocytes (MKs), and the defects in platelet production or function lead to a variety of bleeding complications. Emerging evidence indicates that MKs and platelets are much more diverse than previously appreciated and involved in many physiological and pathological processes besides hemostasis, such as innate and adaptive immune responses, angiogenesis, and tumor metastasis, while the ontogenic variations in MK and platelet function have also become a focus in the field. However, whether MKs and platelets fulfill these distinct functions by utilizing distinct subpopulations remains poorly understood. New studies aimed at deciphering the MK transcriptome at the single-cell level have provided some key insights into the functional heterogeneity of MKs. In this review, we will discuss some of the recent discoveries of functional and developmental heterogeneity of MKs and its potential link to the heterogeneity of platelets. We will also discuss the implications of these findings while focusing on the ex vivo generation of platelets from human pluripotent stem cells. The improved understanding of the heterogeneity underlying human MK development and platelet production should open new avenues for future platelet regeneration and clinical treatment of related diseases.

KEYWORDS

heterogeneity, human pluripotent stem cells, megakaryocytes, platelets

Significance statement

Megakaryocytes (MKs) and platelets have much more diverse functions than previously appreciated, but the exact molecular and cellular features underlying the diverse functions remain largely unclear. This review summarizes the current findings of the functional and developmental heterogeneity of MKs and its potential link with platelet heterogeneity, providing new insights into the ex vivo platelet generation from human pluripotent stem cells and the future clinical treatment of related diseases.

Cuicui Liu and Baiming Huang contributed equally.

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1 | INTRODUCTION

Megakaryocytes (MKs), canonically known as the precursors of blood platelets, are large hematopoietic cells residing mainly in the adult bone marrow (BM) and in the fetal liver (FL) during embryonic development.^{1,2} The current understanding of megakaryopoiesis and thrombopoiesis stems predominantly from the investigation of BM, where they differentiate from hematopoietic stem cells (HSCs), enlarge cell volume by polyploidization, mature and release proplatelets into the vascular lumen that are quickly converted into platelets.^{3,4} Platelets are the smallest anucleate cell member in the blood system essential for preventing hemorrhage.^{5,6}

Emerging evidence from recent years has demonstrated that both MKs and platelets exhibit heterogeneity in morphology, function, and ontogeny. For example, fetal MKs have smaller sizes, lower ploidy, and produce fewer platelets than adult MKs.⁷⁻⁹ Furthermore, MKs appear to possess organ-specific functions far beyond platelet production¹⁰ and can be found in organs such as the lung and spleen.^{11,12} In addition to hemostasis, platelets are also involved in a multitude of inflammatory, antibacterial, angiogenic, and lymphatic processes.¹³ Compared with adults, platelets in developing fetuses have decreased counts and a hyporeactivity toward most agonists. These ontogenic differences may contribute to different functions of platelets.¹⁴

Despite intense efforts, the exact molecular and cellular features underlying the diverse functions of MKs and platelets remain largely unclear. A central question is whether distinct subpopulations of MKs and platelets are dedicated to fulfilling various functions. Although single-cell RNA sequencing has applied to the dissection of cellular heterogeneity and the developmental hierarchy of complicated cell populations, such as erythroid cells^{15,16} and a variety of immune cells,¹⁷ the rarity, fragility, and the difficulty in the isolation of MKs *in vivo* have made the exploration of MKs and human megakaryopoiesis at single-cell resolution extremely challenging. Recently, we and others developed new methodology to isolate MKs *in vivo*, enabling us to unveil distinct MK subpopulations and thereby providing new mechanistic insights into the functional heterogeneity of human MKs.^{7,18,19} This review summarizes the current findings of functional diversity of MKs and platelets, the cellular heterogeneity of MKs, and ontogenic features of MKs and platelets. We also discuss the implications of cellular heterogeneity in platelet production from human pluripotent stem cells (hPSCs) and the future clinical applications.

2 | THE FUNCTIONAL, MOLECULAR, AND CELLULAR HETEROGENEITY OF MKS

Besides the traditional role of MKs as platelet producers, growing evidence has begun to unveil some intriguing details underlying the non-traditional functions of MKs in many physiological and pathological processes, such as HSC quiescence,²⁰ immune regulation,^{21,22} and bone metastasis.²³ For example, MKs in the BM reportedly express multiple immune molecules that may confer their immune regulatory roles including cross-presentation of exogenous antigens, production

of soluble mediators and microparticles, and close interactions with other lineages via emperipoiesis.^{21,23-25} When compared with adult MKs, embryonic and fetal MKs appear to have other functions such as tissue remodeling, which are fulfilled through cytokine secretion and cell-cell interactions.²⁶ Despite the accumulating evidence for the diverse roles of MKs, whether MKs fulfill these distinct functions by specific subpopulations remains mysterious.

To fill these significant gaps in the understanding of human megakaryopoiesis, we have recently decoded human MKs isolated from the yolk sac (YS), FL, and adult BM using single-cell RNA sequencing.^{7,18} Interestingly, we have observed differences in the morphology of MKs in these sites. First, YS MKs are mostly haploid cells with high nucleocytoplasmic ratios and exhibit two distinct morphologies: a fraction of MKs contain numerous cytoplasmic blebs, perhaps in the process of platelet production in a distinctive manner, while the others exhibit less mature features with “smooth” cell membranes. By contrast, a significantly greater proportion of polyploid MKs ($\geq 8N$) is present in human FL than in YS, although the proportion of hyperploidy ($\geq 16N$) MKs is still much lower in FL than in human adults. These observations are consistent with the previous reports^{3,8,9} that human MKs show a shift in cell size, DNA content, and mature stage during ontogenesis (Figure 1, top). Moreover, the immature morphology of lung MKs is observed in both fetal and adult mice.^{22,27} Together, these findings lead us to speculate that morphological differences exist in MKs from different organs and/or in different developmental stages, which are linked to distinct molecular characteristics and functions.

By using contrastive analysis, we have also revealed distinct molecular signatures of MKs from different developmental sites. YS MKs show higher glycolytic features, whereas MKs from FL exhibit higher proliferative characteristics (Figure 1, top).⁷ The distinct metabolic fingerprints and cell cycle signatures are beyond their morphological differences, which might be applied to distinguish human megakaryopoiesis in these two sites. In comparison with embryonic MKs, adult MKs in the BM display more striking hemostatic features, probably indicative of the high potential of platelet generation based on our observations and those from others (Figure 1, top).^{7,18,19} Our findings are in keeping with recent reports that mouse lung MKs exhibit unique immune characteristics that are distinct from mouse FL and BM MKs and skew them toward a role in microbial surveillance and antigen presentation in an MHC II-dependent manner.²² Together, these results are in support of the emerging hypothesis that MKs exert organ-specific functions.¹⁰

In addition, we have also begun to solve a long-standing puzzle and reveal the cellular heterogeneity of both human embryonic and adult MKs for the first time.^{7,18} We show that human embryonic MKs exist as transcriptionally distinct subpopulations with unique patterns of gene expression, which can be linked to early functional specialization (Figure 1). Besides the platelet-forming subpopulation, two separate MK subpopulations with possible niche-supporting and immune functions are identified and may be derived from distinct developmental routes.⁷ Like the embryonic MKs, the adult MKs exhibit similar cellular heterogeneity, and the MK subpopulation with strong immune

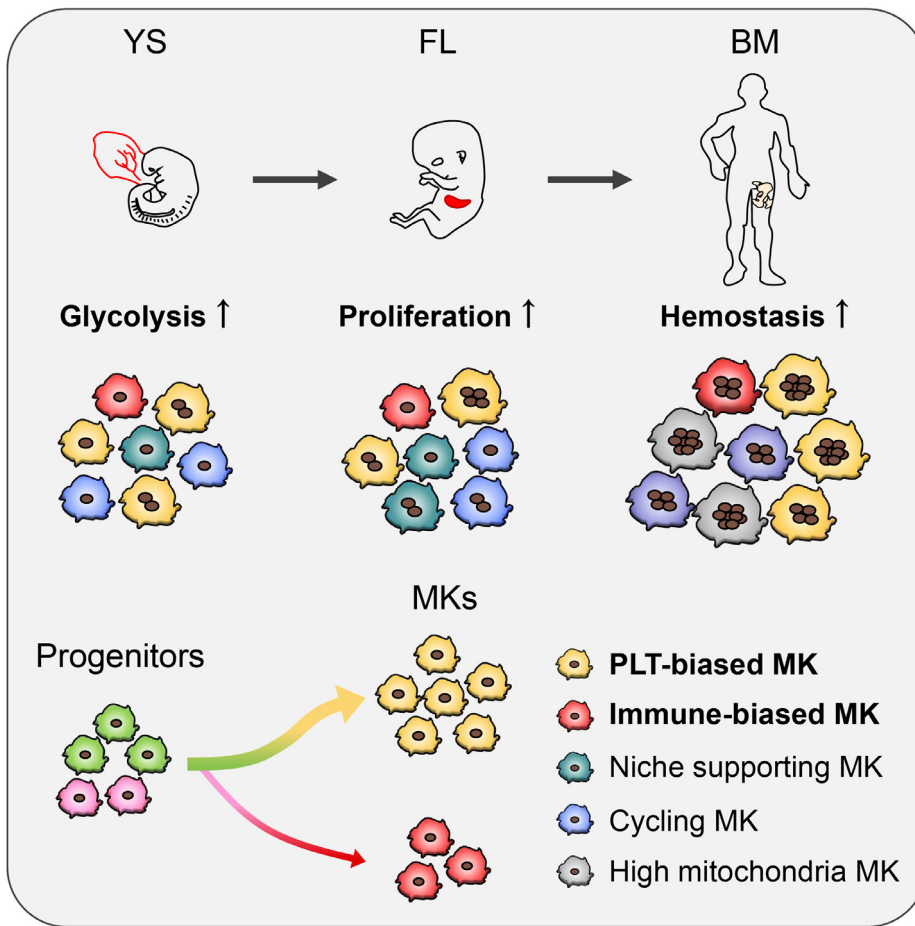


FIGURE 1 The cellular heterogeneity and developmental routes of human MKs. MKs, megakaryocytes

signatures, distinct from platelet-biased MKs, is likely generated from specific progenitors through a unique developmental trajectory (Figure 1, bottom). This immune MK subpopulation, as marked by CD48, exhibits a high-level expression of immune receptors and mediators and might function as immune-surveillance cells (Figure 2).¹⁸ By contrast, the immune subpopulation in human embryonic MKs, which can be distinguished by CD14, expresses genes of the macrophage characteristics at a high level.⁷ The differences in the expression of cellular markers between embryonic and adult immune MKs are probably linked to the evolution in function between them, and this notion can be further explored through a comprehensive analysis of potential spatial and temporal heterogeneity of MKs in future experimentation. Indeed, Sun et al recently uncovered the molecular and spatial heterogeneity within MKs spanning a wide range of ploidy (2N-32N) from mouse BM, which included a unique CD53⁺ MK subpopulation with low-ploidy and a monocytic-like transcriptional program, similar to the lung MKs.¹⁹ Together, these studies provide compelling evidence for the existence of cellular heterogeneity of both embryonic and adult MKs, indicating that the diverse roles of MKs are likely fulfilled by distinct, functionally specialized MK subpopulations rather than by the entire MK population as a whole.

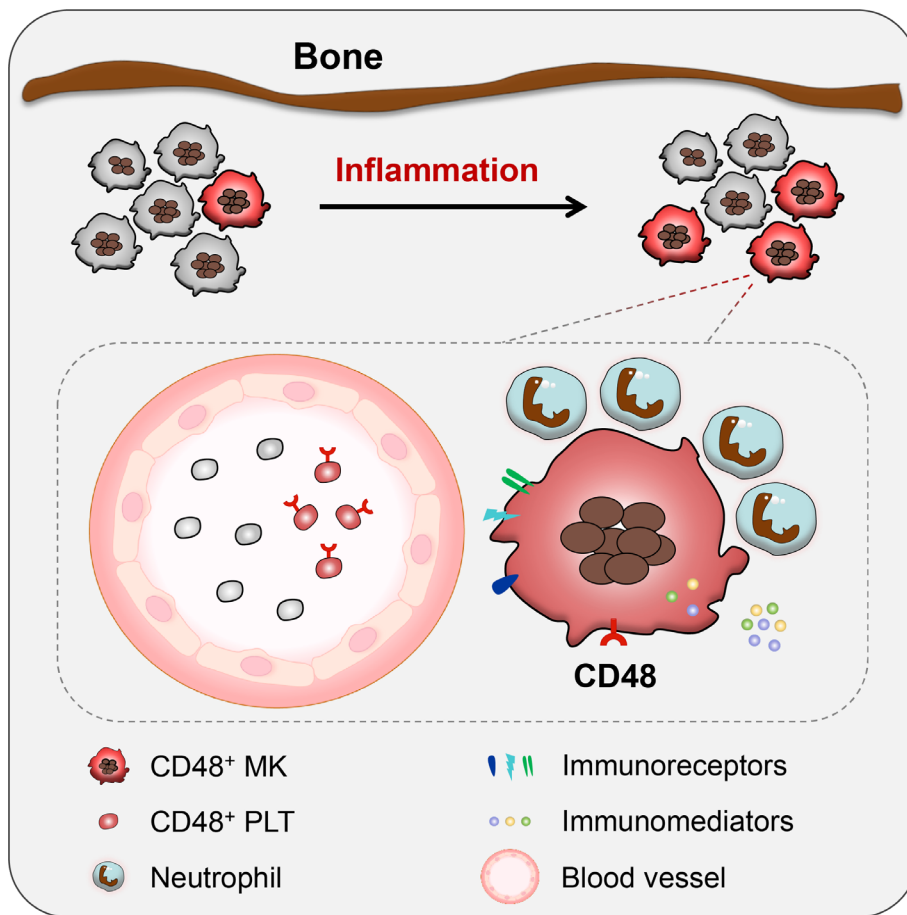
Future efforts should be made to mandate a reclassification of mature MKs in various organs and/or at developmental stages, and this will require further experimentation to detail the functions of

various MK subpopulations under homeostasis or stress conditions and elucidate the events that regulate the differentiation process of various MK subtypes.

3 | THE POTENTIAL LINK BETWEEN MK AND PLATELET HETEROGENEITY

As the progeny of MKs, platelets have even more diverse functions that are far beyond the conventional roles in hemostasis and thrombosis, including the maturation of the lymphatic system and its separation from blood vessels,^{28,29} the acceleration during tumor metastasis and cancer progression,³⁰⁻³² and the effects on both innate and adaptive immune responses through the capacity to interact with almost all known immune cells.^{13,33} For instance, as an important mediator of immune defense, platelets are capable of guiding leukocytes to their sites of extravasation.³⁴ Like many host-defense cells such as dendritic cells and macrophages, platelets can recognize and interact with microbial pathogens via the Toll-like receptors (TLRs) on the surface.³⁵⁻³⁷ More recently, it has been demonstrated that platelets and their parent cells, MKs, can also uptake, process, and present both foreign and self-antigens to CD8⁺ T cells, conferring on them the ability to directly alter adaptive immune responses.^{5,38,39} Moreover, at different developmental stages, platelets also exhibit heterogeneity in

FIGURE 2 The immune-surveillance function of CD48⁺ MKs as illustrated by neutrophil recruitment and platelet release. MKs, megakaryocytes



size, function, life span, and surface receptor expression.⁴⁰ Embryonic platelets, for example, are larger and have weaker reactivity to stimulations than those in adults.¹⁴ In contrast, in elder mammals, platelets show stronger reactivity and better ability to generate thrombus.⁴¹ The platelet diversity during ontogeny may explain the harmful effects during clinical transfusion of “age-mismatched” platelet concentrates.⁴⁰

Although platelets are enucleated and small in volume, the transcriptomic materials inside are diverse and include mRNA coding proteins, microRNAs, and large intergenic non-coding RNAs (lincRNAs), which may play regulatory roles and enable the functional diversity of platelets.^{42,43} However, the links between platelet transcriptome, phenotype, and functions remain concealed in the black box and warrant further investigation. Furthermore, while the current studies of platelet heterogeneity are mainly focused on platelets of different sizes, at various maturation states, and in subjects of different ages,^{14,40} whether platelets contain heterogeneous subpopulations with dedicated functions at a defined state, organ site, and subject and how these distinct subpopulations can be identified are still largely unclear.

The majority of RNA molecules, except those in signal-dependent RNA translation and RNA transfer, and organelles in platelets are inherited from MK precursors.^{32,44} As discussed earlier, MKs exhibit cellular heterogeneity at different developmental stages and in different organs, and it is thus possible that the heterogeneity can be

passed on to the platelets they produce.^{7,27,44} Furthermore, individual MKs may selectively allocate different RNAs or proteins to specific platelets, such as the distribution mode of platelet granules inherited from MKs.⁴⁵ It is reasonable to speculate that the RNA profile in platelets may provide an accessible window for the transcriptional status of MKs at the time of platelet production. Thus, whether the cellular heterogeneity of mature MKs might also lead to the functional heterogeneity of platelets is an intriguing topic for future exploration. Interestingly, we have found that many surface markers expressed in MK subpopulations, such as CD48 in the immune MK subpopulation, can also be detected in platelets, and these platelets increase drastically during inflammatory responses (Figure 2).¹⁸ These observations indicate that the heterogeneity of MKs may directly lead to the heterogeneity of platelets. As such, the diversity of MKs with temporal and spatial characteristics may serve as the main contributor to the developmental and functional heterogeneity of platelets.

As technologies continue to advance, the potential that current roadblocks, such as single-cell sequencing of platelets, can be overcome becomes more and more likely. These technologies will be crucial for delineating the roles of various platelet subpopulations in healthy and diseased states. We thus envision that the identification of age-specific and disease-related platelet subpopulations might have important implications for platelet transfusion and targeted therapy in clinical settings. In combination with other techniques, such as the *in vitro* production of age-matched or function-specific platelet

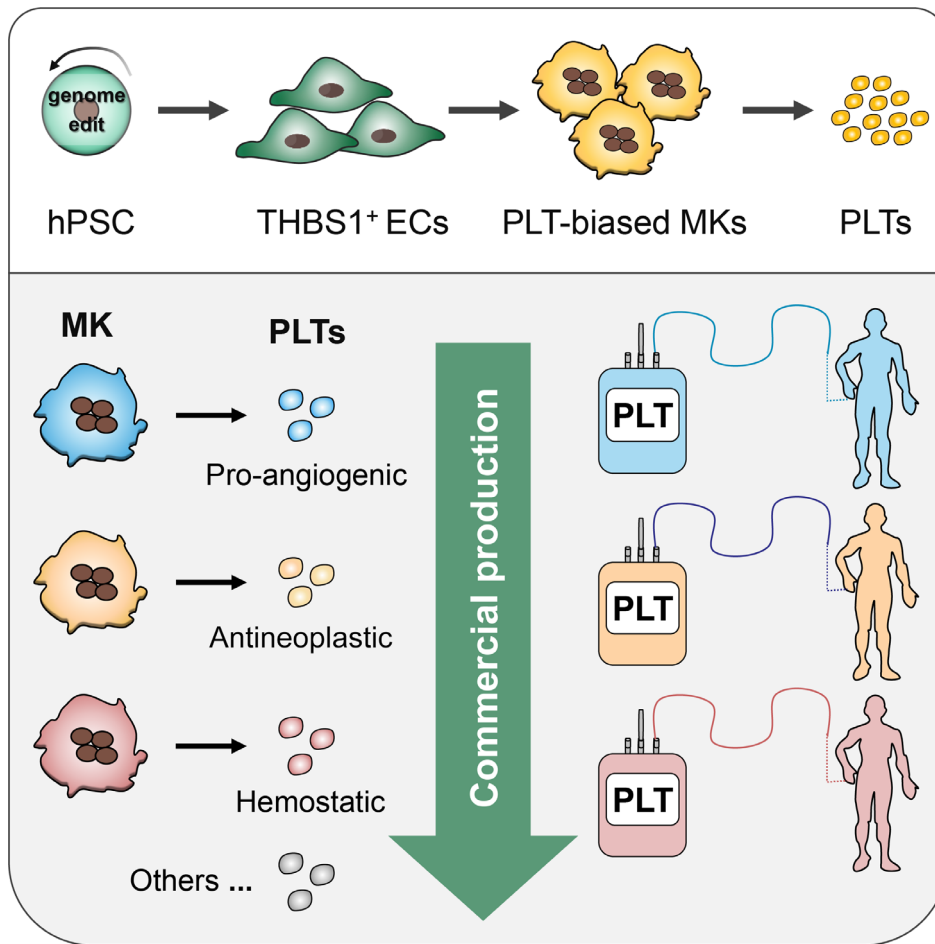


FIGURE 3 New insights into the ex vivo platelet generation and clinical transfusion

concentrates, we might move the clinical application of platelets into a new phase.

4 | NEW INSIGHTS INTO THE PLATELET PRODUCTION FROM hPSCs

Patients with severe thrombocytopenia, platelet functional defects, or undergoing surgery often receive platelet transfusions to reduce the risk of bleeding or treat occurred hemorrhage.⁴⁶ Platelet transfusions are completely donor-dependent, resulting in a limited supply for clinical therapy. hPSCs, including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), have emerged as an effective model for the study of megakaryopoiesis and a promising alternative source to meet the ever-increasing demand for platelet therapy.^{47,48} Several groups including ours have applied gene manipulation, chemical compounds, and modified bioreactors to the development of lab-generated human platelets.⁴⁹⁻⁵³ Recently, Eto and colleagues have made a research breakthrough and produced 200 billion platelets from an hiPSC-based immortalized MK cell line established by introducing three transgenes, c-MYC, BMI1, and BCL-XL, and a bioreactor that provides turbulent energy and shear stress.^{54,55} In addition, Moreau et al have developed a forward programming strategy, relying on the concurrent exogenous expression

of three transcription factors GATA1, FLI1, and TAL1, for the large-scale production of clinically safe and customized platelets from hiPSCs.^{47,48} While a vast majority of the studies have been focused on the improved production of platelets and the subsequent understanding of their classical thrombotic function, very little attention has been paid to the cellular heterogeneity of MKs and platelets produced in vitro from hPSCs.

The identification of distinct MK subpopulations in vivo prompts us to explore the potential MK heterogeneity in vitro. By using the previously established system for the generation of functional MKs and platelets from hESCs,⁵⁶ combined with single-cell RNA-Seq analysis, we show that cellular heterogeneity also exists within the MK population produced from hESCs, thus allowing us to recapitulate early human megakaryopoiesis in vitro.⁷ We find that the platelet-producing and immune-biased subpopulations are all present in hESC-derived MKs, highly reminiscent of the primary MKs from in vivo.⁷ It is, therefore, feasible to induce the formation of specific MK subpopulations in vitro from hPSCs. For platelet production, we envision that the induction of a platelet-biased MK subpopulation may represent a favorable scheme for the generation of highly abundant platelets from hPSCs for clinical applications (Figure 3, top).

Interestingly, by using the hESC-based differentiation system, we have found that endothelial cells (ECs), which serve as the intermediate stage between hESCs and MKs, also contain a subpopulation

biased toward MK differentiation.⁷ Specifically, we identify a subpopulation of thrombospondin1-positive endothelial cells (THBS1⁺ ECs) derived from hESCs, and this subpopulation exhibits elevated potency for megakaryopoiesis and thrombopoiesis following induction.⁷ Our findings suggest that a bias toward MK differentiation might occur at as early as the EC stage during the fate transition from hPSCs to MKs and that megakaryopoiesis may directly emerge from THBS1⁺ ECs. Therefore, THBS1⁺ ECs might represent a new source for producing platelet-biased MKs and subsequently platelets with improved efficiencies and yields (Figure 3, top).

In summary, the elucidation of MK heterogeneity opens new avenues for the subsequent identification of potential platelet subpopulations. An improved understanding of the link between MK and platelet heterogeneity and the underlying molecular basis should make it possible to produce specific subpopulations of platelets with distinct functions *in vitro*. For instance, besides platelets with the canonical function of hemostasis, it would be highly intriguing to produce other potential platelet subpopulations for clinical applications, such as those with antineoplastic or pro-angiogenic functions, from distinct MK subpopulations (Figure 3, bottom).

5 | PROSPECTS

In conclusion, experiments with single-cell profiling of embryonic and adult MKs have provided compelling evidence that MKs fulfill their distinct functions by utilizing heterogeneous subpopulations with devoted responsibilities. As the progeny of MKs, platelets might “inherit” at least in part the expression profiles of receptors or other signaling molecules from MKs, and as such, the cellular heterogeneity of mature MKs may also result in platelet heterogeneity.⁵⁷ The potential of platelet heterogeneity may prove to be extremely attractive for transfusions because it holds the promise to apply separate platelet subpopulations with specific capabilities for a series of clinical problems, such as age-mismatched and ineffective platelet transfusions. We also expect that it will shift the current paradigm of blood group-dependent platelet transfusions and establish new criteria in future clinical applications of platelets. Furthermore, the potential of exploring the transcriptomics of platelets at single-cell resolution should not only expand our understanding of the diverse roles of platelets in diseases but also facilitate the development of therapeutic and diagnostic approaches for related diseases. We envision that the specific platelet subpopulations may be applied for the development of new antithrombotic or antitumor treatments of patients with cardiovascular diseases and various cancers. For example, for targeting malignant diseases, engineered MKs that express chimeric antigen receptors may be induced to differentiate into specific platelet subpopulations to confer additional levels of specificity and precision for immunotherapy.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

C.L., B.H.: data collection, manuscript writing; J.Z., H.W.: conception, manuscript revision.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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