To complicate matters, patient-specific characteristics such as age, absolute lymphocyte count (ALC), and previous therapy can also contribute to the quality of the T-cell product. Nevertheless, CD19 CAR T cells derived from different manufacturing techniques, different lentiviral or retroviral constructs, and different costimulatory moieties have had consistently excellent outcomes in studies targeting children or young adults with ALL.[1,3,4] Thus, discerning which factors are most important for CD19 CAR T-cell persistence and antitumor activity in patients is a challenge. At present, it is not possible to confirm the contention of Gardner et al and thereby justify the more complex and expensive manufacturing process they used, based on patient response rates and survival. Longer-term follow-up on more patients will be necessary to determine whether there is a difference in long-term survival between different CD19 CAR T-cell products.

Another important finding in the article by Gardner et al is the suboptimal expansion and persistence of CD19 CAR T cells in patients with MRD who have low quantities of normal and malignant $CD19^+$ B cells. As the authors point out, lack of the targeted antigen could certainly present a challenge when attempting to incorporate CAR T-cell therapy into first-line therapy or as treatment of MRD after induction or consolidation. Additional means of addressing this potential shortcoming are being explored by several groups and include targeting multiple antigens with CAR T cells or providing additional antigen as a vaccine with CAR T-cell infusion.

Although the multistep manufacturing process used in the Gardner et al study is more complex, it is notable that they report a high success rate in manufacturing with that process, and it is commendable that they report their results on ITT analysis. In some previous studies, it has proved difficult to decipher how many patients were not eligible on the basis of ALC or failure of a test culture.[10] As CD19 CAR T cells move toward licensure, it will be important to streamline the process to reduce the cost of goods and also to determine the standardized ITT response rates with all products to definitively learn whether additional manufacturing maneuvers such as those used by Gardner et al are beneficial. It is an intriguing possibility that a major benefit of the initial separation of the CD4 and CD8 T cells and their independent growth in optimized homeostatic cytokines may be in increasing the manufacturing success rate and making CD19 CAR T-cell therapy available to a greater percentage of patients.

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REFERENCES

1. Gardner RA, Finney O, Annesley C, et al. Intent-to-treat leukemia remission by CD19 CAR T cells of defined formulation and dose in children and young adults. *Blood*. 2017;129(25):3322-3331.
2. Turtle CJ, Hanafi LA, Berger C, et al. Immunotherapy of non-Hodgkin's lymphoma with a defined ratio of CD8+ and CD4+ CD19-specific chimeric antigen receptor-modified T cells. *Sci Transl Med*. 2016;8(355):355ra116.
3. Maude SL, Frey N, Shaw PA, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med*. 2014;371(16):1507-1517.
4. Lee DW, Kochenderfer JN, Stetler-Stevenson M, et al. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. *Lancet*. 2015;385(9967):517-528.
5. Riddell SR, Sommermeyer D, Berger C, et al. Adoptive therapy with chimeric antigen receptor-modified T cells of defined subset composition. *Cancer J*. 2014;20(2):141-144.
6. Sadelain M. Chimeric antigen receptors: driving immunology towards synthetic biology. *Curr Opin Immunol*. 2016;41:68-76.
7. Watanabe N, Bajgain P, Sukumaran S, et al. Fine-tuning the CAR spacer improves T-cell potency. *Oncoimmunology*. 2016;5(12):e1253656.
8. Hudecek M, Sommermeyer D, Kosasih PL, et al. The nonsignaling extracellular spacer domain of chimeric antigen receptors is decisive for in vivo antitumor activity. *Cancer Immunol Res*. 2015;3(2):125-135.
9. Xu Y, Zhang M, Ramos CA, et al. Closely related T-memory stem cells correlate with in vivo expansion of CAR.CD19-T cells and are preserved by IL-7 and IL-15. *Blood*. 2014;123(24):3750-3759.
10. Singh N, Perazzelli J, Grupp SA, Barrett DM. Early memory phenotypes drive T cell proliferation in patients with pediatric malignancies. *Sci Transl Med*. 2016;8(320):320ra3.

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● ● ● HEMATOPOIESIS AND STEM CELLS

Comment on Miyawaki et al, page 3332

Human megakaryocytes: finding the root

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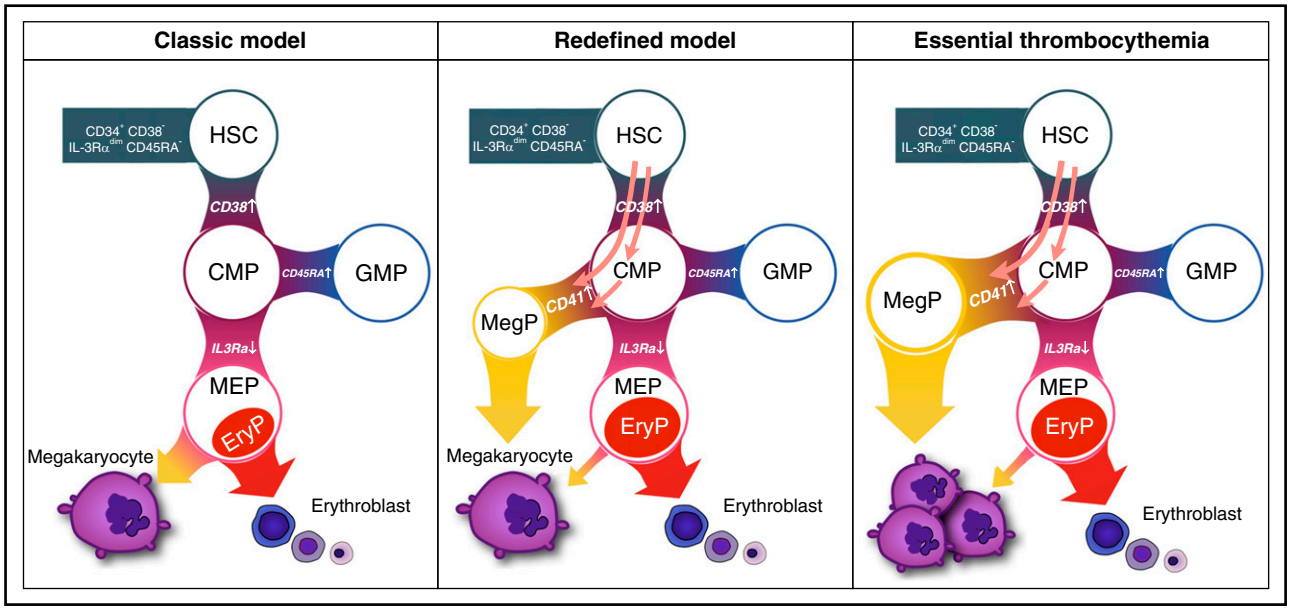
In this issue of *Blood*, Miyawaki et al identify the most primitive progenitor cell population that makes only megakaryocytes and platelets in adult humans and show it is expanded in myeloproliferative neoplasms such as essential thrombocythemia (ET).[1] Approximately 10^{11} platelets are produced on a daily basis in humans, but their exact journey from undifferentiated hematopoietic stem cells (HSCs) is still highly debated. Platelets have the shortest half-life of all blood components and are rapidly recruited when injury occurs, yet have long been thought to be among the cell types to be specified as the furthest from the HSCs in the hematopoietic hierarchy. For several decades, it was understood that differentiation proceeds by a series of binary fates choices, in particular with a common myeloid progenitor (CMP) downstream of HSCs that would give rise to a restricted myeloid progenitor (granulocyte-macrophage progenitor) and to a megakaryocyte-erythrocyte progenitor (MEP). Only downstream of MEPs would unilineage megakaryocyte and unilineage erythrocyte progenitors arise. Recently though, several groups have reported that megakaryocyte and platelet production may not follow this strict hierarchical branching path. Instead, committed megakaryocyte precursors could be found much earlier, either within the HSC[2-4] or the multipotent progenitor compartment.[5] An early precursor that exclusively produces human megakaryocytes in humans, however, had not been described.

Here, Miyawaki et al focused on the human myeloid progenitor compartment and used a combination of single-cell quantitative polymerase chain reaction and in vitro differentiation assays to identify a homogeneous population of cells, which fate has restricted to megakaryopoiesis. For this, they investigated single cells from the classically defined CMP, MEP, and granulocyte-macrophage progenitor



Model of megakaryopoiesis proposed by Miyawaki et al. Megakaryocyte production in human bone marrow proceeds through a unilineage MegP progenitor that can be purified within the classic CMP compartment as CD34$^+$ CD38$^+$ CD45RA$^-$ IL-3Rα^{dim} CD41$^+$. In patients with JAK2 V617F mutated essential thrombocythemia, the MegP population is expanded and may contribute to disease pathology. EryP, unipotent erythrocyte progenitor; GMP, granulocyte-macrophage progenitor. The figure has been adapted from Figure 7 in the article by Miyawaki et al that begins on page 3332.

compartments found in the CD34$^+$ CD38$^+$ fraction of human bone marrow. The only cells that were uniquely committed to produce megakaryocytes in vitro and in vivo (unipotent megakaryocyte progenitors [MegPs]) were found as a distinct subpopulation within the CMP compartment (see figure). This small population (~8% of CMPs) was marked by CD41 cell surface expression and characterized by high expression of the megakaryocyte master transcription factor Fli1. In contrast, only a small percentage of single MEPs would give rise to small megakaryocytic colonies. Interestingly, Miyawaki et al showed that the MegP population was found both in umbilical cord blood and adult bone marrow, suggesting that its presence is maintained over a human lifetime. Of note, CD41$^-$ CMPs could also produce robust platelet engraftment in vivo but always in combination with myeloid and erythroid cells. The key message from this work is that fate restriction to megakaryocyte production predominantly occurs at the level of the CD41$^+$ MegP population in human bone marrow.

A handful of recent studies used single-cell tools to identify when erythroid and megakaryocyte potential separate in humans. Two of these have reported the existence of small pockets of bilineage erythroid-megakaryocyte progenitor cells within the CMP[5] and MEP populations,[5,6] but no unilineage MegPs. The discrepancy between these studies and that of Miyawaki et al is likely to be largely technical, because different sorting strategies and/or cellular sources were used. In addition, genome-wide single-cell RNA-sequencing of the whole bone marrow CD34$^+$ compartment showed strong unilineage megakaryocyte priming in a fraction of the CD34$^+$ CD38$^+$ compartment and the existence of a subpopulation of multipotent progenitors with high contribution to megakaryopoiesis.[7] What emerges is a picture in which, at the single-cell level, the classically defined CMP and MEP are much more heterogeneous than originally thought. Specifically, it seems that the proportion of cells that have capacity to differentiate into more than one mature blood cell type is very likely low, especially in adults. Similar conclusions were recently reached in mouse models.[8,9]

Two questions that remain open are: How are MegPs specified at the molecular level? And which cell is their direct parent? The authors demonstrated that CD41$^-$ CMPs could give rise to CD41$^+$ CMPs in vitro, but whether this is the main route of MegP production in vivo at homeostasis or following injury remains to be clarified. This may be difficult to assess directly in human cells with the current tools, but, because studies in mice have suggested that in certain conditions HSCs exclusively give rise to platelets[2,4] and that megakaryocyte-restricted progenitors can reside in the compartment that is commonly used to purify HSCs,[3,10] it would be interesting to know if the MegP is an obligatory cellular step during megakaryopoiesis.

Another important finding of this study is that MegPs isolated from patients with JAK2 V617F mutant ET are expanded and tend to produce more megakaryocytes than those from healthy controls (see figure). Direct comparison of the function of JAK2 V617F mutated and wild-type MegPs and their progenitors within the same patient was not carried out. However, one implication of these data is that mutations such as JAK2 V617F, recurrent in myeloproliferative neoplasms or in clonal hematopoiesis of indeterminate potential, can have strong effects not only on the HSC compartment but also on specific progenitor cell types. In the case of expansion of MegPs in ET, future studies will have to address how much this expansion accounts for ET pathology and identify which molecular mechanisms downstream of the JAK2 V617F mutation drive this expansion specifically in patients with ET and not in patients with other types of myeloproliferative neoplasms. A deep understanding of the normal routes of commitment, their fluctuations over a human lifetime, and how they are skewed in malignancy, will be highly informative for how we should treat blood diseases.

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REFERENCES

1. Miyawaki K, Iwasaki H, Jiromaru T, et al. Identification of unipotent megakaryocyte progenitors in human hematopoiesis. *Blood*. 2017;129(25):3332-3343.
2. Sanjuan-Pla A, Macaulay IC, Jensen CT, et al. Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy. *Nature*. 2013;502(7470):232-236.
3. Haas S, Hansson J, Klimmeck D, et al. Inflammation-induced emergency megakaryopoiesis driven by hematopoietic stem cell-like megakaryocyte progenitors. *Cell Stem Cell*. 2015;17(4):422-434.
4. Shin JY, Hu W, Naramura M, Park CY. High c-Kit expression identifies hematopoietic stem cells with impaired self-renewal and megakaryocytic bias. *J Exp Med*. 2014;211(2):217-231.
5. Notta F, Zandi S, Takayama N, et al. Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. *Science*. 2016;351(6269):aab2116.
6. Psaila B, Barkas N, Iskander D, et al. Single-cell profiling of human megakaryocyte-erythroid progenitors identifies distinct megakaryocyte and erythroid differentiation pathways. *Genome Biol*. 2016;17:83.
7. Velten L, Haas SF, Raffel S, et al. Human haematopoietic stem cell lineage commitment is a continuous process. *Nat Cell Biol*. 2017;19(4):271-281.
8. Perié L, Duffy KR, Kok L, de Boer RJ, Schumacher TN. The branching point in erythro-myeloid differentiation. *Cell*. 2015;163(7):1655-1662.
9. Paul F, Arkin Y, Giladi A, et al. Transcriptional heterogeneity and lineage commitment in myeloid progenitors [published correction appears in Cell. 2016; 164:325]. *Cell*. 2015;163(7):1663-1677.
10. Roch A, Trachsel V, Lutolf MP. Brief report: single-cell analysis reveals cell division-independent emergence of megakaryocytes from phenotypic hematopoietic stem cells. *Stem Cells*. 2015;33(10):3152-3157.

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● ● ● HEMATOPOIESIS AND STEM CELLS

Comment on Fares et al, page 3344

EPCR: a novel marker of cultured cord blood HSCs

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In this issue of *Blood*, Fares et al[1] demonstrate that endothelial protein C receptor (EPCR) is a reliable marker of human cord blood (CB) hematopoietic stem cells (HSCs), both from uncultured cells and those expanded with UM171, a pyrimidoindole derivative previously shown to expand CB HSCs.[2]

These studies build on 2 prior observations, that exposure of CB-derived $CD34^+$ cells to UM171 leads to a rapid induction of EPCR expression both at the messenger RNA and protein levels[2] and that EPCR expression can be used to identify murine HSCs,[3,4] the latter finding suggesting that EPCR may be used similarly in the human setting. Identifying methods to quickly assess expansion of human HSCs ex vivo is an important issue, as many groups have developed methods to expand CB hematopoietic stem and progenitor cells (HSPCs) ex vivo (reviewed in Mehta et al[5]); however, as cultured HSCs frequently exhibit altered surface phenotypes,[6,7] surrogate markers that reliably define HSCs expanded ex vivo need to be identified because assessment of long-term (LT) HSC activity from expanded cultures currently relies on time-consuming serial transplantation experiments in immunocompromised mice. Identifying markers that can identify HSCs rapidly would facilitate the development of high-throughput screening strategies to assess novel HSC ex vivo expansion methods as well as the identification of molecular pathways involved in HSC function.

In the present study, the authors demonstrate that only $ECPR^+CD34^+$ CB cells express 2 previously identified human HSC markers, CD90 and CD133, indicating that EPCR expression defines a subset of HSCs with a more primitive phenotype ($CD34^+CD38^-CD49f^{med}CD90^+CD133^+$).[7-9] Given the ability of EPCR to enrich for LT-HSCs in unmanipulated CB cells, the authors also tested whether EPCR identifies LT-HSCs in expanded CB cultures. The enrichment in repopulating activity among $EPCR^+$ cells increased during culture from day 7 (20-fold relative enrichment, $EPCR^+$ vs bulk) to day 12 (56-fold). Indeed, using limiting dilution analyses, the authors showed that UM171-expanded cultures from day 7 cultures contained $EPCR^+$ cells enriched for functional multipotent LT-HSCs (1 in 68 cells) compared with $EPCR^{low}$ cells (1 in 2016) or $EPCR^-$ cells (1 in 4240). In addition, the authors confirmed that CB-enriched HSPCs are hierarchically organized, with $EPCR^+CD34^+$ cells giving rise to $EPCR^-CD34+$ cells, but not vice versa. $EPCR^+CD34^+$ cells also exhibited self-renewal, as only $EPCR^+$ cells were able to engraft secondary recipients. Together, these studies showed that EPCR marks functional HSCs.

To determine if EPCR is essential for HSPC function, EPCR was silenced with targeted short hairpin RNA vectors or ectopically expressed. Although EPCR has been shown to regulate mouse LT-HSC bone marrow (BM) and mice expressing low levels of EPCR ($Procr^{low}$) showed defects in HSC BM homing associated with increased levels of circulating HSCs,[10] the authors showed that loss or overexpression of EPCR had no impact on the expression of other HSC markers, cell proliferation, or the ability to repopulate NOD-Scid IL-2Rγ null (NSG) mice, suggesting that EPCR may not be required for HSPC function in humans (see figure). To determine the molecular differences among ex vivo expanded CB cells, the authors compared the transcriptomes of $ECPR^-$, $EPCR^{low}$, and $EPCR^+$ cell fractions sorted from UM171-treated $CD34^+CD45RA^-$ CB cells. $EPCR^+$ cells were enriched for HSC-associated genes compared with $EPCR^{low/-}$ cells, and the authors defined a 120-gene signature associated with EPCR expression composed of transcriptional regulators such as *HLF*, *PRDM16*, and *MECOM*, as well as genes encoding antigens previously described as expressed by HSCs including CD90, CD133, and GPR56. Collectively, these data demonstrate that EPCR is a novel marker that allows the identification and prospective separation of human LT-HSCs, both in unmanipulated and ex vivo UM171 expanded CB HSCs.

Although these studies represent an exciting advance in HSC biology, it will be important for others to confirm these results and test whether EPCR also can be used to prospectively separate HSC-enriched populations from $CD34^+$ cells from adult BM and granulocyte-colony stimulating factor mobilized peripheral blood, particularly because it was previously reported that EPCR may not mark human BM-derived HSCs.[3] In