RESEARCH ARTICLE SUMMARY

HEMATOPOIESIS

Distinct routes of lineage development reshape the human blood hierarchy across ontogeny

Faiyaz Notta,* Sasan Zandi,* Naoya Takayama, Stephanie Dobson, Olga I. Gan, Gavin Wilson, Kerstin B. Kaufmann, Jessica McLeod, Elisa Laurenti, Cyrille F. Dunant, John D. McPherson, Lincoln D. Stein, Yigal Dror, John E. Dick†

INTRODUCTION: The hematopoietic road map is a compilation of the various lineage differentiation routes that a stem cell takes to make blood. This program produces greater than 10 blood cell fates and is responsible for generating more than 300 billion cells daily. On several occasions over the past six decades, the murine road map has been reconceived due to new information overturning dogma. However, the human road map has changed little. In the human model, blood differentiation initiates at the level of multipotent stem cells and passes through a series of increasingly lineage-restricted oligopotent and, finally, unipotent progenitor intermediates. One critical oligopotent intermediate is the common myeloid progenitor (CMP), believed to be the origin of all myeloid (My), erythroid (Er), and megakaryocyte (Mk) cells. Although murine studies challenge the existence of oligopotent progenitors, a comprehensive analysis of human My-Er-Mk differentiation is lacking. Moreover, whether the pool of oligopotent intermediates is fixed across human development (fetal to adult) is unknown.

RATIONALE: The differentiation road map taken by human hematopoietic stem cells (HSCs) is fundamental to our understanding of blood homeostasis, hematopoietic malignancies, and regenerative medicine.

RESULTS: We mapped the cellular origins of My, Er, and Mk lineages across three time points in human blood development: fetal liver (FL), neonatal cord blood (CB), and adult bone marrow (BM). Using a cell-sorting scheme based on markers linked to Er and Mk lineage specification (CD71 and CD110), we found that previously described populations of multipotent progenitors (MPPs), CMPs, and megakaryocyte-erythroid progenitors (MEPs) were heterogeneous and could be further purified. Nearly 3000 single cells from 11 cellular subsets from the $CD34^+$ compartment of FL, CB, and BM (33 subsets in total) were evaluated for their My, Er, and Mk lineage potential using an optimized single-cell assay.

In FL, the ratio of cells with multilineage versus unilineage potential remained constant in both the stem cell ($CD34^+CD38^-$) and progenitor cell ($CD34^+CD38^+$) enriched compartments. By contrast, in BM, nearly all multipotent cells were restricted to the stem cell compartment, whereas unilineage progenitors dominated the progenitor cell compartment. Oligopotent progenitors were only a negligible component of the human blood hierarchy in BM, leading to the inference that multipotent cells differentiate into unipotent cells directly by adulthood.

Mk/Er activity predominantly originated from the stem cell compartment at all developmental time points. In CB and BM, most Mks emerged as part of mixed clones from HSCs/MPPs, indicating that Mks directly branch from a multipotent cell and not from oligopotent progenitors like CMP. In FL, an almost pure Mk/Er progenitor was identified in the stem cell compartment, although less potent Mk/Er progenitors were also present in the progenitor compartment. In a hematological condition of HSC loss (aplastic anemia), Mk/Er but not My progenitors were more severely depleted, pinpointing a close physiological connection between HSC and the Mk/Er lineage.

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CONCLUSION: Our data indicate that there are distinct road maps of blood differentiation across human development. Prenatally, Mk/Er lineage branching occurs throughout the cellular hierarchy. By adulthood, both Mk/Er activity and multipotency are restricted to the stem cell compartment, whereas the progenitor compartment is composed of unilineage progenitors forming a "two-tier" system, with few intervening oligopotent intermediates. ■

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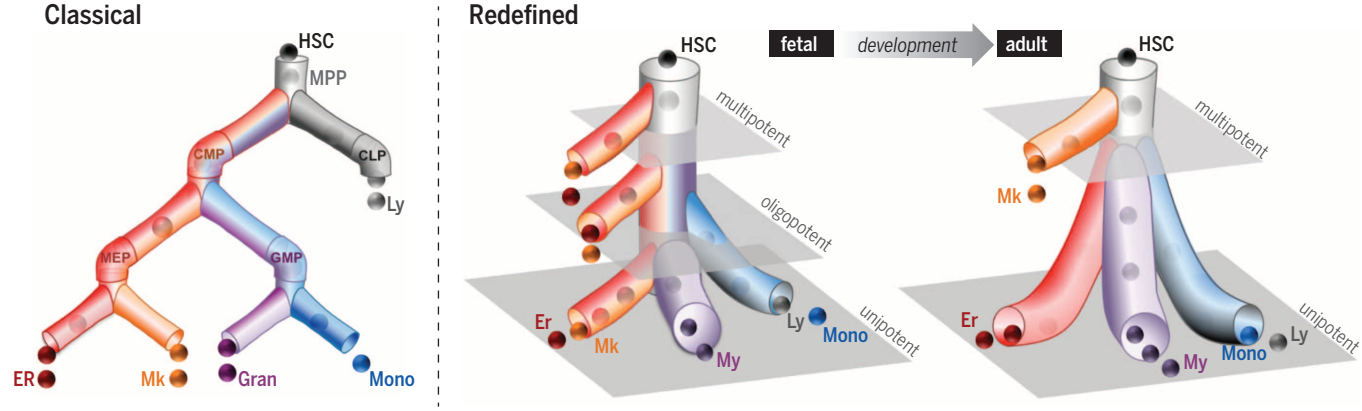
Road maps of human blood stem cell differentiation. The classical model envisions that oligopotent progenitors such as CMP are an essential intermediate stage from which My/Er/Mk differentiation originates. The redefined model proposes a developmental shift in the progenitor cell architecture from the fetus, where many stem and progenitor cell types are multipotent, to the adult, where the stem cell compartment is multipotent but the progenitors are unipotent. The grayed planes represent theoretical tiers of differentiation.



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Distinct routes of lineage development reshape the human blood hierarchy across ontogeny

Faiyaz Notta,[1,2]*† Sasan Zandi,[1,2]* Naoya Takayama,[1,2] Stephanie Dobson,[1,2] Olga I. Gan,[1] Gavin Wilson,[2,4] Kerstin B. Kaufmann,[1,2] Jessica McLeod,[1] Elisa Laurenti,[6] Cyrille F. Dunant,[7] John D. McPherson,[3,4] Lincoln D. Stein,[2,4] Yigal Dror,[5] John E. Dick[1,2]‡

In a classical view of hematopoiesis, the various blood cell lineages arise via a hierarchical scheme starting with multipotent stem cells that become increasingly restricted in their differentiation potential through oligopotent and then unipotent progenitors. We developed a cell-sorting scheme to resolve myeloid (My), erythroid (Er), and megakaryocytic (Mk) fates from single CD34$^+$ cells and then mapped the progenitor hierarchy across human development. Fetal liver contained large numbers of distinct oligopotent progenitors with intermingled My, Er, and Mk fates. However, few oligopotent progenitor intermediates were present in the adult bone marrow. Instead, only two progenitor classes predominate, multipotent and unipotent, with Er-Mk lineages emerging from multipotent cells. The developmental shift to an adult "two-tier" hierarchy challenges current dogma and provides a revised framework to understand normal and disease states of human hematopoiesis.

For decades, both human and mouse hematopoiesis has been described as a cellular hierarchy maintained by self-renewing hematopoietic stem cells (HSCs) that reside at the apex of its pyramidal structure of differentiating cells (*1*, *2*). This differentiation scheme highlights key features of the blood hierarchy that has been critical to our understanding of how stem cells manage lifelong blood production. In general, self-renewing cell types with extended life span like long-term HSCs (LT-HSCs), as well as short-term HSCs (ST-HSCs) and multipotent progenitors (MPPs), are rare and remain closer to the conceptual peak of the hierarchy; oligopotent and unipotent progenitors farther down the scheme have shorter life spans, increase numerically, and ultimately differentiate into more than 10 functional blood cell types. In the standard model of hematopoiesis, hierarchical differentiation commences from HSCs with the production of stem cell intermediates with less durable self-renewal potential that culminate with the generation of MPPs, the penultimate step before lineage specification. From MPPs, the common lineages for myelopoiesis [common myeloid progenitor (CMP)] and lymphopoiesis [common lymphoid progenitor (CLP)] are segregated (*3*, *4*). In myeloid (My) (defined herein as granulocyte/monocyte) differentiation, oligopotent CMPs undergo further restriction into bivalent granulocyte-monocyte progenitor (GMPs) that go on to make granulocytes and monocytes, and megakaryocyte-erythroid progenitors (MEPs) that go on to make platelets and red blood cells (RBCs). Thus, CMPs represent the critical oligopotent progenitor from which all My, erythroid (Er), and megakaryocyte (Mk) cells arise. Although the standard model is still used extensively as an operational paradigm, further cell purification and functional clonal assays have led to key revisions to the model. In mouse, the identification of lymphoid-primed multipotent progenitors (LMPPs) argued that Mk-Er potential must be the first lineage branch lost in lymphomyeloid specification of HSCs (*5*, *6*). Recently, paired-daughter analysis monitoring of mouse HSC cell divisions have demonstrated that Mk-Er progenitors can be derived from HSC directly without progressing through conventional MPPs and CMPs (*7*). Although these data challenge the standard model, clear consensus on a revised model of hematopoiesis is still lacking. Human hematopoiesis is widely regarded as following the same differentiation scheme as mouse hematopoiesis [reviewed in (*8*)]. Early work involving cell purification and methylcellulose (MC) colony-forming cell (CFC) assays identified CMPs and CLPs (*9*, *10*). However, purification schemes to resolve My, Er, Mk, and lymphoid (Ly) fates remained poor. Through the development of more efficient assays to monitor Ly fates in single-cell stromal assays and an improved sorting scheme, we identified human multilymphoid progenitors (MLPs) as the earliest lymphoid differentiation precursor with concomitant lymphoid (T, B, and natural killer) and myelomonocytic potential (*11*, *12*). Considerable uncertainty remains concerning the myelo-erythro-megakaryocytic branch of human hematopoiesis because clonogenic CFC assays do not read out My, Er, and Mk fates efficiently or contemporaneously, making it difficult to account for all cells within phenotypically pure populations of CMPs and MEPs. A comprehensive analysis of human myelo-erythro-megakaryocytic development has not been undertaken, so it is really only by default that the standard model applies.

Much of our understanding of the molecular basis of cellular differentiation and lineage commitment is derived from the assumptions implicit in the standard model. For example, simultaneous expression of molecular factors associated with My-Er-Mk lineages at low levels is considered to maintain CMPs as the origin of the common lineage for myelopoiesis (*3*). During lineage restriction to GMPs and MEPs, progressive upregulation of particular lineage factors initiates feedforward and feedback molecular controls that lock in a granulocyte/monocyte or a Mk-Er differentiation program. An important axiom that arises from this molecular view of the standard model is that cellular differentiation is gradual. However, transcriptional studies of highly purified or single-cell murine HSC has established that molecular programs corresponding to My-Er-Mk fates can directly emerge in multipotent cells, arguing that cellular differentiation is not gradual and that myeloid differentiation can occur without progressing through an intermediate CMP stage (*6*, *7*, *13*–*17*). Naik *et al.* have demonstrated that nearly half of the LMPP compartment is biased toward dendritic cell commitment, a lineage previously thought to come from the CMP-to-GMP route (*15*). Molecular factors associated with Mk-Er differentiation have been shown to be active in LT-HSCs (*13*, *14*), and prospective isolation of platelet-biased LT-HSCs strongly supports that this lineage is not derived from the CMP to MEP route (*16*). Whether molecular programs that regulate My-Er-Mk fates arise at the level of HSCs in humans is not known. It is important to understand where Er and Mk lineage branching occurs in the human hematopoietic hierarchy because these lineages comprise 99% of the cellular component of blood and represent the bulk of the 300 billion blood cells that turn over daily in humans. Mapping the cellular origins of Er and Mk lineages in the human blood hierarchy represents a critical step to define the molecular basis of their fate commitment.

The cellular road map describing the blood hierarchy has been built on two core experimental pillars: cell purification and clonal assays. Human

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studies reporting on sorting schemes for the myelo-erythroid progenitor hierarchy (CMPs, GMPs, and MEPs) have often assumed that "marker-pure" subsets are synonymous with "functionally pure" subsets (*9*). In other words, each cell within the purified subset possesses the same differentiation potential (Fig. 1A, 1). This interpretation is primarily derived from clonogenic assessment of human CMPs, GMPs, and MEPs using standard CFC assays. In CFC assays, purified CMPs typically generate My, Er, or Mk colonies; GMPs give rise to My colonies only; and MEPs give rise to Er and/or Mks (*9*). Because CMPs displayed all lineage readouts, whereas GMPs and MEPs did not, CMPs were interpreted as both marker-pure and functionally pure on the basis that the CFC assay was inefficient in being able to read out mixed differentiation potential. This reasoning underpins the basic bifurcating scheme of human CMPs to GMPs and MEPs. However, an alternate interpretation exists if we assume that the CFC assay is actually efficient. In this case, human CMPs are phenotypically homogenous (e.g., marker-pure) but are functionally heterogeneous, consisting of diverse unipotent progenitors (Fig. 1A, 2). This contention could only be proven if a new sorting strategy is able to isolate, in functionally pure form, each type of the unilineage progenitor from the starting CMP population. To distinguish between the two alternatives, we need (i) a new sorting strategy for human myelo-erythroid progenitors, and (ii) a more sensitive assay to assess mixed cell potential. Until both scenarios can be experimentally resolved, there is considerable uncertainty that clouds the classical view of the human hematopoietic hierarchy. We attempted to address both of these issues by developing a cell-sorting scheme and an optimized single-cell assay to efficiently read out My, Er, and Mk fates from putative multilineage cell types.

Results

Previously defined human MPPs, CMPs, and MEPs are heterogeneous

We developed a cell-sorting scheme to examine the cellular heterogeneity within the CD34$^+$ compartment of human blood. To a previous seven-parameter design (CD34, CD38, CD7, CD10, FLT3, CD45RA, and Thy1) (*11*), we distinguished HSCs from MPPs by adding CD49f (*18*); identified Er-Mk progenitors by adding cMPL (CD110) (*19*, *20*) (designated here as BAH1 to be consistent with the antibody clone used to detect this antigen) (*21*, *22*) and CD71 (transferrin receptor); and distinguished B-lymphoid–committed progenitors from My progenitors, such as GMPs in the CD45RA$^+$ fraction, by adding CD19. Upon evaluation in human fetal liver (FL), neonatal cord blood (CB), and adult bone marrow (BM), this 11-parameter cell-sorting layout provided a high-resolution view of the phenotypic heterogeneity that exists within CD34$^+$ cells across all developmental stages (Fig. 1B and fig. S1).

Many distinct cell types, such as HSCs, MPPs, and MLPs, reside within the CD34$^+$CD38$^-$/lo (simplified as CD34$^+$CD38$^-$ herein) stem cell–enriched compartment of human blood. We investigated whether CD71 or BAH1 expression corresponded to a known cell type within this compartment in the FL. About 10% of the FL CD34$^+$CD38$^-$ expressed CD71, and half of these CD71$^+$ cells also expressed BAH1 (fig. S1A, 2). Neither CD71 nor BAH1 was expressed on Thy1$^+$ HSCs (fig. S2A) or on CD45RA$^+$ MLPs (fig. S2B), which suggests that these markers identify a different cell type within the MPP compartment. We redefined the current MPP compartment into three fractions (F1, F2, and F3) on the basis of CD71 and BAH1 expression: MPP F1 cells were CD71$^-$BAH1$^-$, MPP F2 cells were CD71$^+$BAH1$^-$, and MPP F3 cells were CD71$^+$BAH1$^+$ (Fig. 1B, vii). The expression of these molecules in the CD34$^+$CD38$^-$ compartment was unexpected because the onset of Mk-Er lineage commitment according to the standard model occurs at the level of CMPs and MEPs that are found in the progenitor-enriched CD34$^+$CD38$^+$ compartment. These data suggest that the detection of functional Er and Mk differentiation molecules on a subset of CD34$^+$CD38$^-$ cells represents a unique Mk-Er branch point within the multilineage compartment.

We next analyzed CD71 and BAH1 expression in the CD34$^+$CD38$^+$ progenitor compartment. Co-expression of FLT3 and the lymphoid antigen CD7 in FL CD34$^+$CD38$^+$ cells (fig. S2C) indicated that CD7 expression is not exclusive to lymphoid progenitors as reported previously in CB (*23*). Thus, CD7-expressing cells were not excluded in our sorting layout. In lieu of CD7, we used CD10 expression to exclude Ly progenitors (Fig. 1B, i; fig. S1, 6). In the FL CD34$^+$CD38$^+$CD10$^-$ cell compartment, FLT3 and CD45RA expression

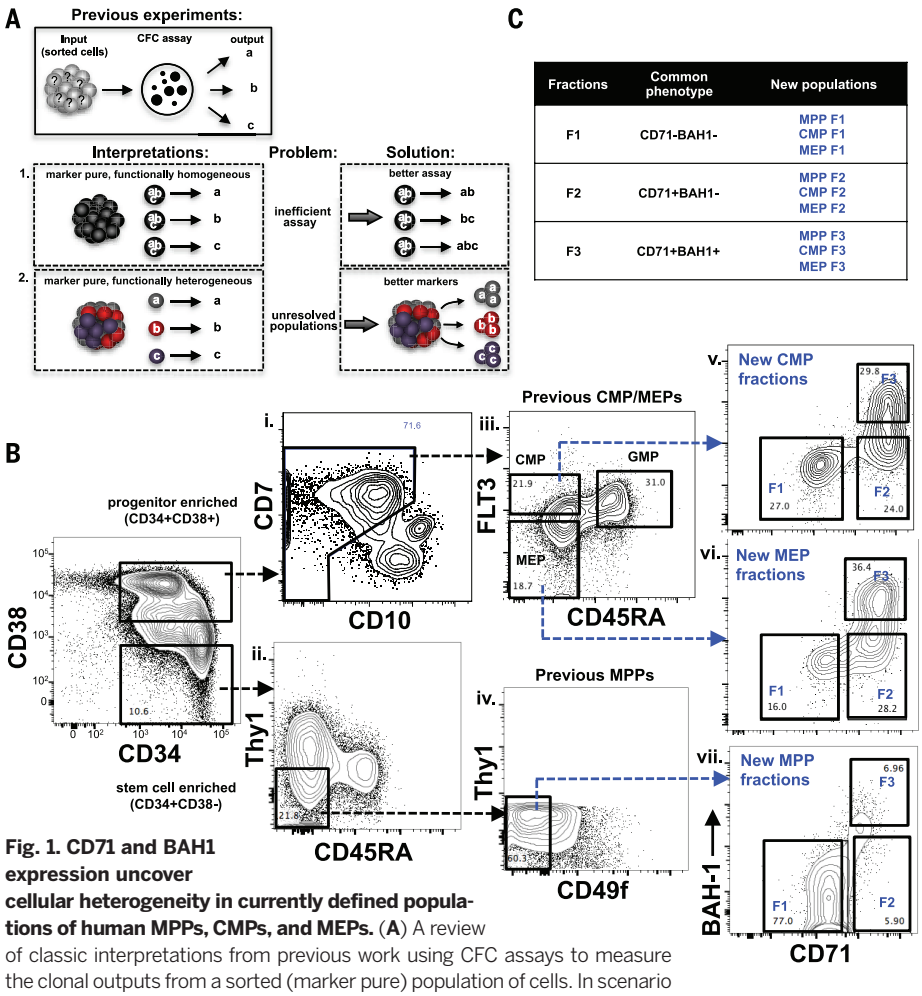

Fig. 1. CD71 and BAH1 expression uncover cellular heterogeneity in currently defined populations of human MPPs, CMPs, and MEPs. (**A**) A review of classic interpretations from previous work using CFC assays to measure the clonal outputs from a sorted (marker pure) population of cells. In scenario 1, each cell within a marker-pure population has the potential to give rise to three functional outputs (a, b, and c) but only gives rise to one of them in the assay. Under this condition, diverse functional outputs from a marker-pure population are interpreted to be derived from a functionally homogeneous population of multilineage cells. In the alternate scenario 2, a marker-pure population is composed of three distinct unipotent cell types that give rise to lineages a, b, and c independently. Both of these scenarios can be reconciled with a better assay (right top) or better markers (right bottom). (**B**) The gating scheme of defining MPPs (CD34$^+$CD38$^-$Thy1$^-$CD45RA$^-$CD49f$^-$), CMPs (CD34$^+$CD38$^+$CD10$^-$FLT3$^+$CD45RA$^-$), and MEPs (CD34$^+$CD38$^+$CD10$^-$FLT3CD45RA$^-$) from a representative human FL sample is shown (black dashed arrows). MPPs, CMPs, and MEPs were further divided into F1 (CD71$^-$BAH1$^-$), F2 (CD71$^+$BAH1$^-$), and F3 (CD71$^+$BAH1$^+$) (blue dashed arrows). Full gating scheme for FL, CB, and BM CD34$^+$ cells is presented in fig. S1. (**C**) Summary of the new subsets used in this study.

was used to identify commonly defined CMPs ($FLT3^+CD45RA^-$), GMPs ($FLT3^+CD45RA^+$), and MEPs ($FLT3^-CD45RA^-$) (Fig. 1B, iii; fig. S1, 7). Addition of CD71 and BAH1 to CMP and MEP populations uncovered phenotypic heterogeneity within these populations previously considered to be homogeneous. In line with the nomenclature we used for the redefined MPP compartment described above, CMP and MEP compartments were also subdivided into three fractions with CD71 and BAH1 (F1, $CD71^-BAH1^-$; F2, $CD71^+BAH1^-$; F3, $CD71^+BAH1^+$) (Fig. 1B, v to vii, and fig. S1, panels 8 and 10). We repeated the same analysis in CB and adult BM to determine whether CB and adult BM samples were similarly heterogeneous. All the major cell populations identified in the FL were also observed in CB and BM, albeit to different degrees (table S1), indicating that the cellular heterogeneity uncovered by CD71 and BAH1 existed across all developmental stages (fig. S1, B and C). Thus, previously defined human CMPs and MEPs that were considered homogeneous are in fact phenotypically heterogeneous when Er and Mk markers are applied.

In summary, previously defined MPPs, CMPs, and MEPs contain three distinct cellular fractions: F1, lacking CD71 and BAH1; F2, expressing CD71 but lacking BAH1; and F3, expressing both molecules. A total of 33 distinct cellular classes from FL, CB, and BM (11 per developmental stage) were functionally interrogated to evaluate their lineage fate potential. To facilitate the review of the results below, a legend and complete phenotype is provided in Fig. 1C and table S1.

An optimized single-cell assay for human My-Er-Mk progenitors

To evaluate the functional potential of the cellular subsets identified above, we developed a single-cell in vitro assay that overcame the shortcomings of previous approaches to characterize human myeloid progenitors. An ideal assay would support the ability of single cells to simultaneously commit along My, Er, and/or Mk fates, as well as to provide the conditions for their differentiated progeny to survive, propagate, and expand to permit detection. Standard MC assays do not strictly fulfill these criteria. For example, $CD49f^+$ HSCs exclusively generated CFU-My in MC (fig. S3A and supplementary text), yet single HSCs sustain multilineage hematopoiesis in vivo (*18*). Also, the limited self-renewal potential of downstream progenitors makes them difficult to read out in vivo at clonal resolution, further highlighting the need to develop more sensitive in vitro methodology to assess the lineage potential of progenitors. We found that serum-free conditions supplemented with growth factors (SCF, FLT3, TPO, EPO, IL-6, IL-3, IL-11, GM-CSF, and LDL) and stroma were highly efficient at assaying My, Er, and Mk lineage potential from single $CD34^+$ cells. Single-cell–derived clones were analyzed by flow cytometry after a 2- to 3-week culture period for Mk (CD41 and CD42b), Er (GlyA), and My (CD14, CD15, and CD33) cells. One example of the efficiency of this new assay comes from the analysis of the $CD49f^+$ HSC subset, which previously could not be read out in vitro as single cells (*11*). Under these new conditions, 77% of FL, 72% of CB, and 48% of BM single $CD49f^+$ HSCs were able to produce a clone (fig. S5A, left). Whereas only CFU-My were produced from HSC in MC, mixed clonogenic potential was now readily detectable with this assay. We used this assay to functionally map the lineage potential of all the newly defined $CD34^+$ subsets from all three developmental time points.

Unipotent progenitors dominate the blood hierarchy by adulthood

To gain a global perspective of the functional differences in the blood hierarchy across ontogeny, we first combined all 11 $CD34^+$ subsets from each developmental time point into one analysis of nearly 3000 single cells. Cloning efficiency was highest for FL and decreased gradually in CB and BM (FL, 74%; CB, 69%; BM, 55%) (Fig. 2A). A simple stratification based on whether a single cell gave rise to one (unilineage) or more (multilineage) cell lineages revealed that 40% of FL $CD34^+$ cells were multilineage compared with 27% of CB ($P < 10^{-3}$, Fisher's exact test) and 18% of BM ($P < 10^{-3}$, Fisher's exact test) $CD34^+$ cells (Fig. 2B). Thus, the ratio of multilineage to unilineage progenitors changes en bloc in development within the $CD34^+$ population, a result that is independent of the complex marker scheme that we used.

To continue exploring the organizational relationships of progenitors across developmental time points, we investigated the proportion of multilineage to unilineage cells in the stem-cell–enriched ($CD34^+CD38^-$) and the progenitor-enriched ($CD34^+CD38^+$) subsets. Within $CD34^+CD38^-$ cells, FL and CB had a significantly higher proportion of multilineage cells compared with BM (FL versus BM: 48.6% versus 32.9%, $P = 0.0016$; CB versus BM: 46.1% versus 32.9%, $P = 0.011$; Fisher's exact test). These proportional differences were more pronounced in the $CD34^+CD38^+$ progenitor compartment. In $CD34^+CD38^+$ cells, BM displayed a factor of 3 fewer multilineage cells compared with FL (FL, 28.8%; CB, 17.3%; BM, 9.6%; $P < 0.0001$, Fisher's exact test) (Fig. 2, C and D). Thus, both the stem and progenitor compartments from each developmental stage exhibited a proportional

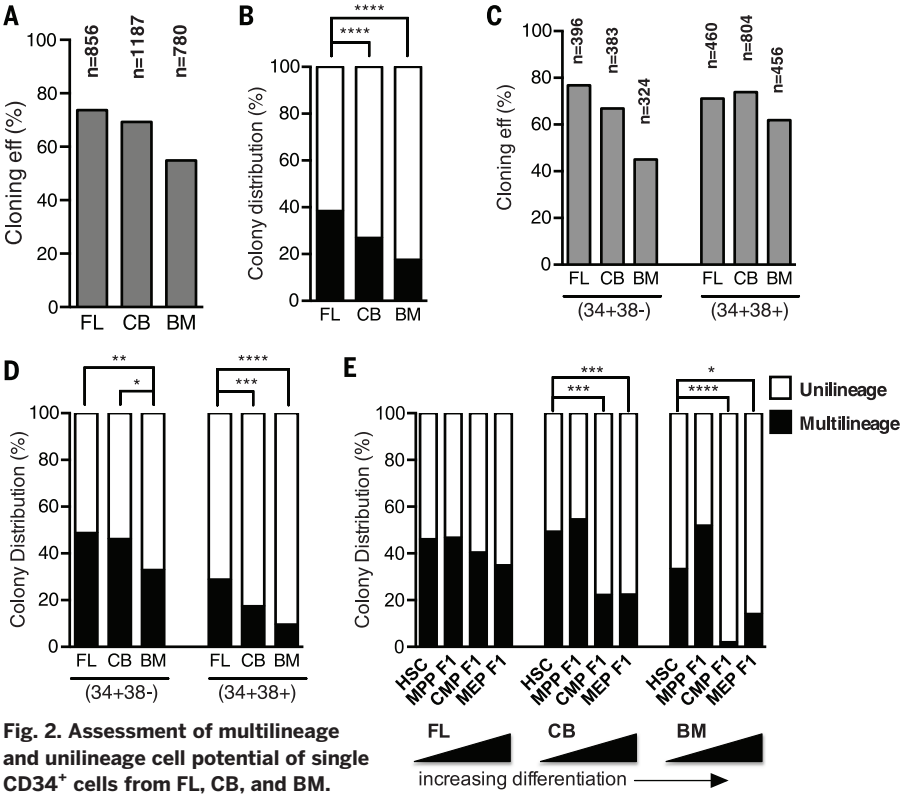

Fig. 2. Assessment of multilineage and unilineage cell potential of single $CD34^+$ cells from FL, CB, and BM. (**A** to **D**) Single cells from subsets defined in Fig. 1C were deposited by fluorescence-activated cell sorting (FACS) and cultured for several weeks. Emergent clones were analyzed by flow cytometry for My, Er, and Mk lineages (Fig. 3A). To gain a global perspective of the functional differences between FL, CB, and BM, subsets were combined into one analysis of $CD34^+$ cells [(A) and (B)] or stem ($CD34^+CD38^-$) and progenitor ($CD34^+CD38^+$) cell compartments [(C) and (D)]. A single cell was defined as multilineage (black) when it gave rise to more than one lineage (any two of My, Er, or Mk) and unipotent when it gave rise to one lineage (My or Er or Mk) [(B) and (D)]. Overall cloning efficiency is shown in gray [(A) and (C)]. (**E**) Distribution of multilineage and unilineage cell potential from populations that lack CD71 and BAH-1 (F1) expression (shown by increasing differentiation: HSC > MPP F1 > CMP F1 > MEP F1). Asterisks indicate significance based on Fisher's exact test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

change in the percentage of multilineage cell types.

Next, we localized the differentiation stages most affected by the loss of multilineage progenitors. We reasoned that HSCs and subsets that lack differentiation markers CD71 and BAH1 (MPP F1, CMP F1, and MEP F1) would be enriched for cells with multilineage cell potential. Notably, in FL, the ratio of multilineage to unilineage progenitors remained nearly constant across these subsets (Fig. 2E). By contrast, only HSC and MPP F1 subsets from CB and BM were highly enriched for multilineage cells, whereas their corresponding CMP/MEP F1s were composed mostly of unilineage cell types (Fig. 2E; $P < 0.05$, Fisher's exact test). In BM, virtually all multilineage cells were restricted to the $CD34^+CD38^-$ stem cell compartment (Fig. 2E; $P < 0.05$, Fisher's exact test). Hence, multilineage cell potential extends into the progenitor compartment in FL, but in BM, this potential is restricted to the $CD34^+CD38^-$ stem cell compartment. In parallel, the progenitor compartment in BM is dominated by unilineage cell types.

Mk-Er lineage branching in the blood hierarchy is developmentally defined

To gain a detailed understanding of the differentiation potential of each progenitor subset identified by our sorting scheme applied to FL, CB, and BM, we classified the functional potential of each single cell in our data set. Five distinct clonal outputs were classified: Mk only, Er only, My only, Mk/Er, and mixed (bipotent, Er/My or Mk/My; tripotent, Er/Mk/My) (Fig. 3A). The cloning efficiency of all subsets was high in MPP, CMP, and MEP fractions (50 to 80%) (Fig. 3B and fig. S5A).

The highest percentage of mixed clones was found among the HSC subsets (FL, 46.1%; CB, 49.3; BM, 33.3%) (fig. S5A), except in BM, where MPP F1 harbored higher mixed clone potential than HSC (51.9%), although this was not significant (BM HSC versus MPP F1: $P = 0.1$, Fisher's exact test) (Fig. 2E). FL HSC and MPP F1 subsets had a statistically higher distribution of tripotent versus bipotent mixed clones compared with CB and BM HSC and MPP F1 (fig. S5B). In FL and CB, Mk-Er-only clones appeared at the MPP F1 stage (FL, 29% of total; CB, 18% of total) (Fig. 3B, column 1, row 1). In BM, Mk-Er clones from MPP F1 were rare (~2%); rather, 15% of all clones from this subset were Er-only. Mk activity from BM MPP F1 was detected as a component of mixed clones that also contained My cells (further discussed below).

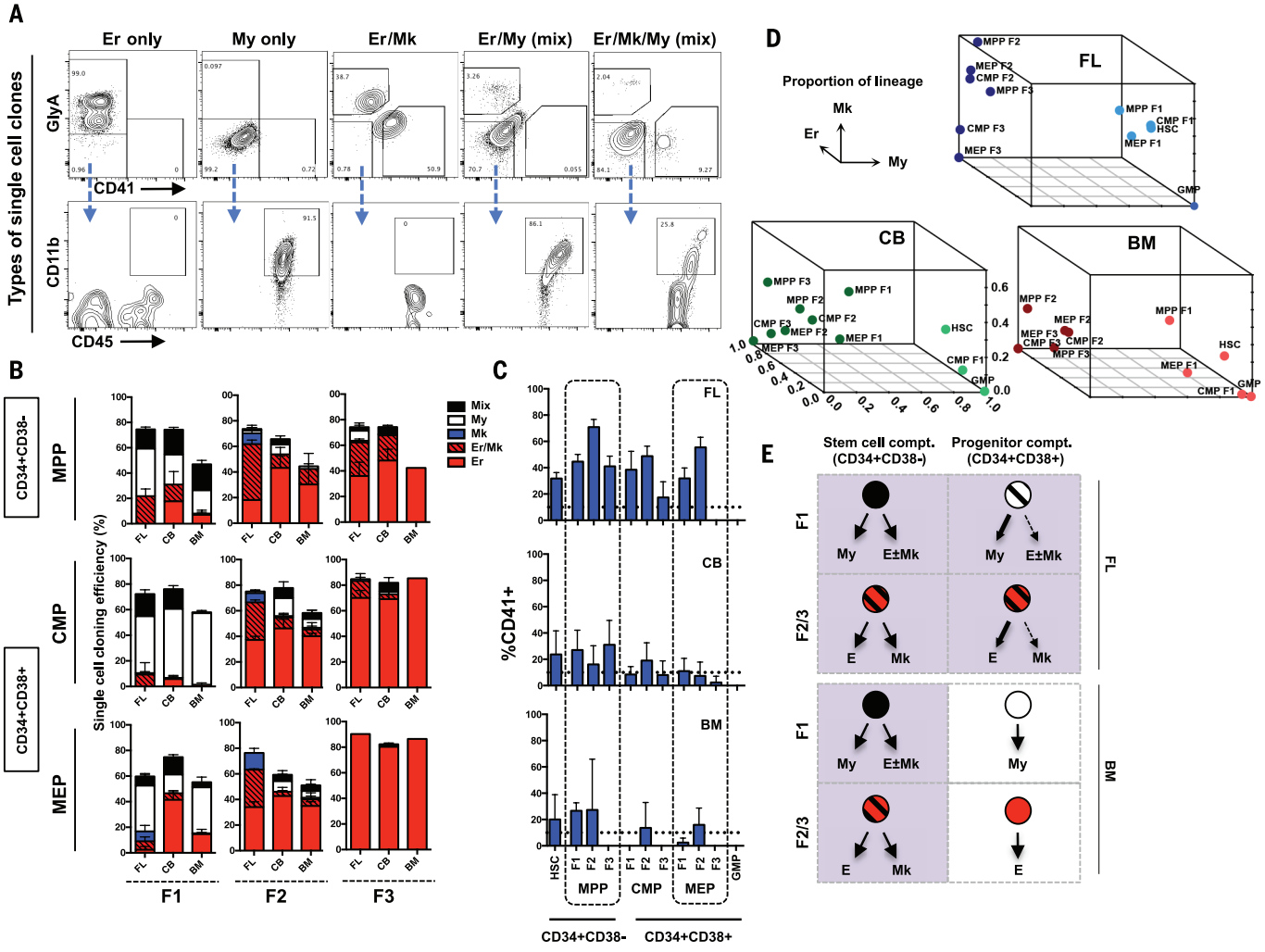

Fig. 3. Lineage analysis of single-cell clones from subfractions of MPP, CMP, and MEP populations. (**A**) Single-cell clones were analyzed by flow cytometry and binned into five distinct lineage outcomes. Erythroid clones were defined as $GlyA^+$ only (Er only). Myeloid clones were identified as $GlyA^-CD41^-$ but $CD45^+CD11b^+$ (My only). Erythroid-megakaryocyte clones were defined as $GlyA^+$ and $CD41^+$ but negative for CD11b (Er/Mk). Mix clones were defined My and Er or Mk (column 4) or My, Er, and Mk (column 5). (**B**) Cloning efficiency and lineage outcomes of single cells from newly defined MPP, CMP, and MEP fractions (F1, F2, and F3) from FL, CB, and BM. (**C**) Total Mk output ($CD41^+$) from all newly defined subsets from FL, CB, and BM. Bars indicate mean ± SE. Total number of independent experiments: $n = 3$, 6, and 4 for FL, CB, and BM, respectively. The dotted line defines the threshold for detecting positive Mk lineage potential from a sorted fraction due to analytical noise that arises from single clone flow cytometry. (**D**) Three-dimensional summary of lineage outputs (My, Er, and Mk) from all cellular subsets in FL, CB, and BM presented in (B). (**E**) Pictorial depiction of the predominant lineage outcomes from stem ($CD34^+CD38^-$) and progenitor ($CD34^+CD38^+$) cell compartments in FL and BM data.

We next interrogated the $CD34^+CD38^+$ compartment. Based on the classical view of the blood hierarchy, we would expect that true CMPs reside in a subset that lacks expression of differentiation markers such as CD71 and BAH1 (CMP F1). Only 15% of FL and CB CMP F1 clones were mixed, and no mixed clones from this subset were detected in BM (Fig. 3B, column 1, row 2). Thus, we conclude that previously defined BM CMPs are not homogeneous for cells with multilineage My-Er-Mk potential; rather, they are heterogeneous and composed of subpopulations of unilineage My, Er, and Mk progenitors (Fig. 3B, row 2). To determine whether the small percentage of mixed clones from FL and CB CMPs F1 population were derived from bona fide CMPs, we evaluated their My-Er-Mk potential. More than 80% of the mixed clones from FL and CB were bipotent (either Er/My or Mk/My) without concurrent Mk-Er-My potential (fig. S5C). These data demonstrate that bona fide CMPs are a rare component of the human hematopoietic tree, irrespective of developmental stage.

MC and megacult colony assays indicated that subsets defined by CD71 and BAH1 expression (F2 and F3) were highly enriched for Mk and Er activity (fig. S3, A to D, and supplementary text). However, these assays cannot formally rule out that Er and Mk potential was derived from independent unilineage progenitors. We first tracked Mk-Er activity from single cells within the MEP subsets (MEP F1 through F3) (Fig. 3B, row 3). MEP F1 was highly heterogeneous across developmental time points and composed mostly of My progenitors in FL and BM (60% or more) (Fig. 3B, column 1, row 3) that functionally resemble other F1 subsets from MPPs and CMPs. In CB and BM, ~70% of clones from MEP F2 were Er-only, and 10% or less were Mk-Er clones (Fig. 3B, column 2, row 3). MEP F2 is likely the subset within classical MEPs that gave rise to low-level Mk colonies in previous studies. MEP F3, the numerically dominant cell population within the classical MEPs, uniformly produced Er-only clones in FL, CB, and BM (Fig. 3B, column 3, row 3). Thus, classically defined MEPs are principally composed of Er-only progenitors when analyzed at single-cell resolution and are not Mk-Er progenitors as previously thought.

As only rare cells within the MEP fractions give rise to Mks, Mk potential must lie elsewhere in the blood hierarchy. We found that most Mk-Er activity came from the $CD34^+CD38^-$ stem cell compartment (fig. S3C) and was particularly enriched within one of our newly defined MPP subsets (Fig. 3B, column 2, row 1). In FL, 60% of clones from MPP F2 were of Mk-Er type, and the remainder of this subset was composed of Mk-only or Er-only clones (Fig. 3B, column 2, row 1). Notably, Mk activity was enriched but not restricted to the stem cell compartment in the FL. Because we did not find strong evidence for FL CMPs, we expect that FL Mk-Er progenitors arise from the stem cell compartment, specifically from MPP F2. In CB and BM, Mk-Er clones represented one-quarter of the total clonal output from this subset (Fig. 3B, column 2, row 1), and the rest were Er-only clones. In CB—and more evident in BM—Mks predominantly emerged as part of mixed clones from HSCs and MPP F1, supporting the hypothesis that Mk branching occurs directly from a multipotent cell, as predicted by the murine studies (*7*). These data suggest that both Mk-Er and multilineage potential are restricted to the stem cell compartment by adulthood, whereas unilineage fates predominate the progenitor compartment, forming a simple "two-tier" hierarchy, with few intervening oligopotent intermediates (Fig. 3, D and E).

In vivo analysis establishes hierarchical relationships between progenitor subsets

In the blood hierarchy, cell types near the peak of the hierarchy, such as HSCs and MPPs, are rarer but possess higher proliferative potential. Lineage differentiation typically correlates with loss of proliferative potential. Although HSC and MPP subsets (F1 to F3) are minor populations, they yielded 5 to 10 times as many cells compared with more abundant populations from the CMP and MEP subsets in vitro (fig. S6A).

We then transplanted our cellular subsets in vivo and measured graft durability and size, as well as its lineage composition, to establish the hierarchical relationships of our newly defined progenitor subsets. Due to tissue availability, only CB was used. Because there are limited data on the engraftment capacity of human progenitors in the NOD-*Scid-Il2rg*^{null} (NSG) model, we first scrutinized the repopulation kinetics of human blood cells in this model. Using HSCs, we observed low-level lymphomyeloid as well as erythro-megakaryocytic engraftment as early as 2 weeks after transplant (fig. S6, B and C), consistent with previous studies using NOD-*Scid* mice (*24*). We used 2 weeks as the standpoint from which to assess progenitor cell engraftment in vivo. One thousand CMP F1 cells and 3000 MEP F1 cells generated a myelo-erythroid restricted graft that did not persist beyond 2 weeks (Fig. 4A, column 3 and 5, and fig. S6E). By contrast, 200 MPP F1 cells were able to sustain a robust and systemic multilineage graft (My-Er-Ly) beyond 2 weeks, consistent with the functional potential of true MPPs (Fig. 4A, column 1; Fig. 4B; and fig. S6E) (*18*).

To gather enough cell numbers for in vivo detection of Er-enriched subsets, we combined the F2 and F3 subsets from MPPs, CMPs, and MEPs for transplantation because they shared similar functional potential in vitro. The combined F2/F3 subsets from MPPs, CMPs, and MEPs all gave rise to prominent Er grafts in vivo, concordant with their in vitro potential (Fig. 4A, columns 2, 4, and 6; and fig. S6D, top panels). MPP F2/F3 cells were highly proliferative and generated a robust Er graft with only 400 transplanted cells

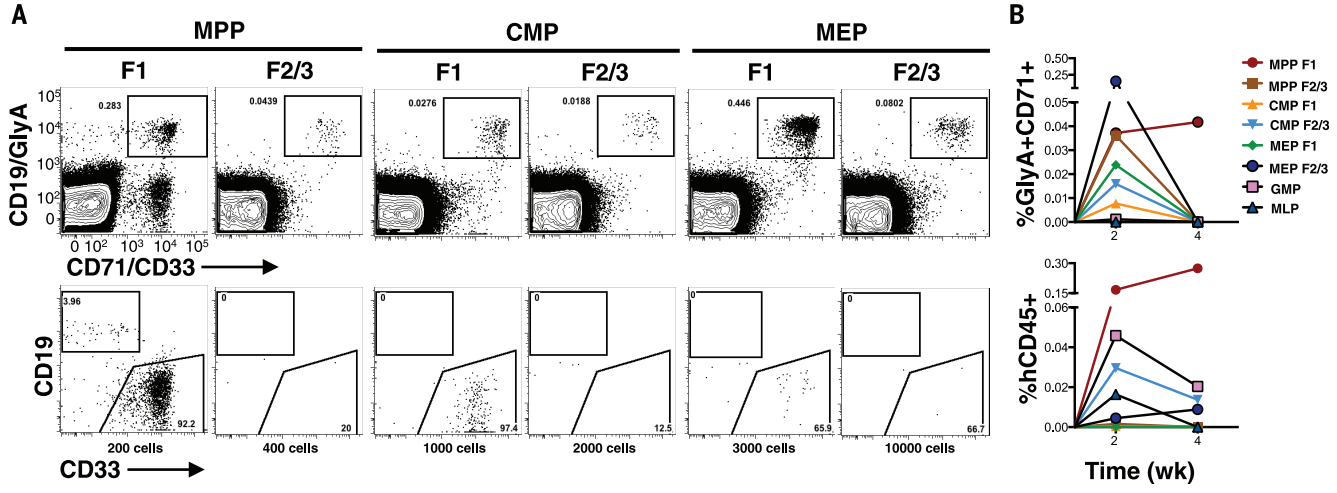

Fig. 4. In vivo potential of progenitor subsets. (**A**) Freshly sorted populations from CB were intrafemorally transplanted into sublethally irradiated NSG mice. Bone marrow from injected femur and noninjected bones were analyzed by flow cytometry 2 weeks after transplant. The average transplanted cell dose is shown at the bottom of the flow plot. Top row indicates Er engraftment ($GlyA^+CD71^+$). Bottom row indicates total human leukocyte engraftment ($CD45^+$). B-lymphoid cells and My cells were detected using CD19 and CD33, respectively. (**B**) Kinetic analysis of engraftment from progenitor subsets. Mean levels of Er ($GlyA^+CD71^+$) and total human cell engraftment ($CD45^+$) are shown.

(Fig. 4A, column 2), whereas CMP F2/F3 and MEP F2/F3 required cell doses higher by a factor of 5 to 25 to generate an in vivo graft (Fig. 4A, columns 4 and 6). Although platelets were difficult to detect in vivo from progenitor subsets, we did observe them in rare mice engrafted with either MPP F1 or MPP F2/3 cells (fig. S6C). Only MPP F2/F3, but not CMP F2/3 and MEP F2/3, were able to migrate systemically to nontransplanted bones and resemble the proliferative potential of MPP F1 (fig. S6E). When combined with the in vitro analyses of these subsets, these in vivo experiments support that Mk-Er-enriched MPP F2/F3 are derived from HSCs or MPPs directly without invoking a lineage route via a CMP intermediate.

A transcriptionally defined erythroid progenitor subnetwork in the CD34 hierarchy

Lineage commitment coincides with the expression of key molecules that aid to "lock in" a differentiation program (*25*). Using low-cell-input RNA-sequencing methodology (*26*), we first analyzed the expression profile of canonical lineage factors in bulk CB subsets. Genes associated with My specification, such as *MPO* and *CSF2RA* (*GM-CSFR*), were highly expressed in GMPs, whereas genes associated with the Er lineage, such as *GATA-1* and *EPOR*, were highly expressed in F2/F3 subsets from MPP, CMP, and MEP populations (fig. S7A). Mk differentiation markers, *CD41* (*ITGA2B*) and *CD42b* (*GP1BA*), were highly expressed in MPP F2, in line with the functional potential of this subset. These data provide an independent line of evidence that committed Mk progenitors reside within the stem cell compartment. Low-level expression of *CD41* and *CD42b* was also detected in MPP F3 and MEP F2, consistent with the residual Mk activity from these CB subsets (fig. S7A).

Unsupervised hierarchical clustering and principal component analysis of the entire data set revealed two major molecular subgroups: those with Er-enriched potential (MPP, CMP, and MEP F2/F3), and another with multilineage (HSC or MPP F1) or My-enriched potential (CMP F1 or MEP F1) (fig. S7B). F2 and F3 subsets within MPP, CMP, and MEP populations clustered together, suggesting that these pairs are more closely related within each broader compartment (fig. S7Bi). Due to their close transcriptional and functional relationship, we merged F2 and F3 subsets from MPP, CMP, and MEP subsets to compare global transcriptional differences among these Er-enriched subsets. We found that 230 genes were differentially expressed between MPP (F2/F3) versus CMP (F2/F3), and 52 genes between CMP (F2/F3) versus MEP (F2/F3). These differentially expressed genes were highly enriched in the cell cycle and DNA replication and metabolic processes, and coincide with the extensive proliferation that Er progenitors undergo during specification (fig. S7C). These data reinforce the idea that the close functional relationship between Mk-Er and Er enriched subsets is likely due to shared molecular programs.

Molecular heterogeneity among single cells within a purified subset is commonly lost in a population-level analysis. We tracked the expression of key lineage factors among single cells in our progenitor subsets. Only FL and BM were used in this experiment because they represent the two ends of the development time points used in this study. In both FL and BM, single cells from F2 and F3 from MPPs, CMPs, and MEPs displayed a dominant Er gene expression program, often co-expressing both *GATA1* and *EPOR* genes in the same cell (Fig. 5A and fig. S7D). Our sorting method

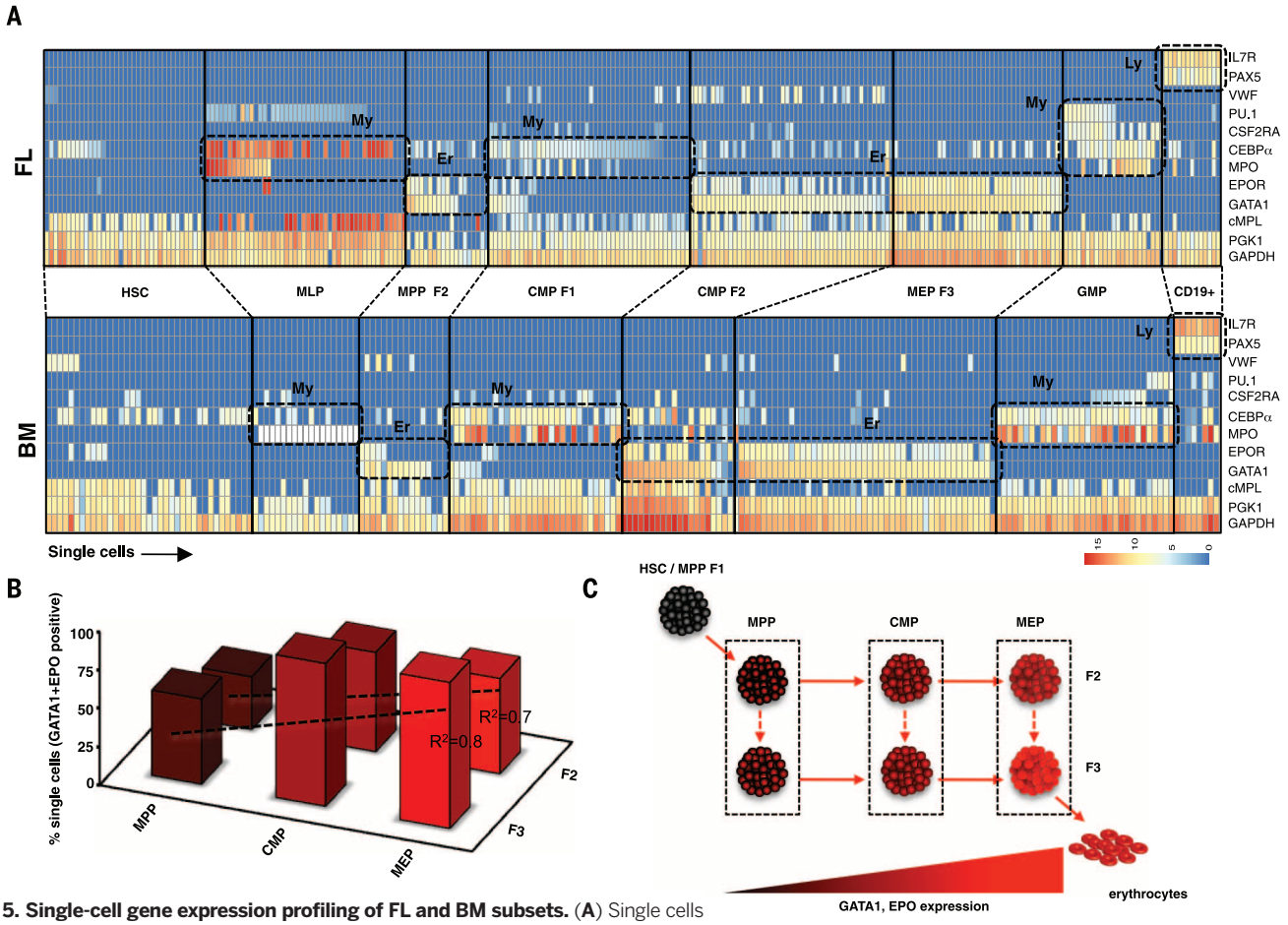

Fig. 5. Single-cell gene expression profiling of FL and BM subsets. (**A**) Single cells from FL (top) and BM (bottom) subsets were sorted and analyzed for expression of genes associated with My, Er, and Ly lineages (shown on right) on the Fluidigm platform. My, Er, or Ly gene clusters are shown as dashed boxes. (**B**) Percentage of single cells that coexpress *GATA1* and *EPOR* in F2 and F3 from MPP, CMP, and MEP subsets. BM and FL F2 and F3 subsets were combined in this analysis. (**C**) A theoretical subnetwork of Er progenitors within the $CD34^+$ compartment.

can essentially resolve the Er-committed progenitors within the CD34$^+$ hierarchy. We observed that *GATA1* expression was present among most single cells in MPP F2 and MPP F3, but *EPOR* expression was only present in a subset of *GATA1*-positive cells (Fig. 5, A and B). Because *GATA1* precedes *EPOR* expression in Er differentiation, MPP F2/F3 cells represent the earliest erythroid differentiation precursor in the human blood hierarchy. The percentage of single cells that co-expressed *GATA1* and *EPOR* increased in proportion among F2 and F3 subsets from MPPs, CMPs, and MEPs in both FL and BM (Fig. 5B). These molecular factors are considered a surrogate of the degree of Er differentiation (Fig. 5B). When considered jointly with our in vitro and in vivo analyses, these subsets are already Er-specified but vary mostly in their proliferative potential. We hypothesize that these subsets compose a hierarchical subnetwork of Er progenitors within the CD34$^+$ compartment (Fig. 5C). A candidate network map is shown in Fig. 5C. Because erythrocytes comprise nearly 99% of all blood cells, this network may offer a high degree of flexibility to synthesize erythrocytes under homeostatic and emergency erythropoiesis without HSC input.

Dramatic loss of Er progenitors compared with My progenitors in a hematologic condition of HSC deficiency

Recent HSC fate-mapping analyses in mice have provocatively shown that progenitors, but not HSCs, are fundamental for ongoing hematopoiesis under homeostatic conditions (*27*, *28*). Physiological studies to experimentally test this question are not possible in normal human subjects. However, certain disease states permit a glimpse into the consequences of HSC loss on progenitors and may shed light on the role of HSCs in human blood synthesis under nontransplant conditions. In aplastic anemia (AA), HSCs are damaged, likely due to an autoimmune response, and are unable to contribute to ongoing hematopoiesis (*29*, *30*). This effect seems to be specific to HSCs, because all mature blood lineages are depressed in AA (*31*–*33*). We examined the progenitor hierarchy in three cases of AA by applying our sorting scheme (Fig. 6). Consistent with previous reports, the proportion of CD34$^+$ cells within the overall mononuclear cell (MNC) pool was significantly lower in AA compared with normal BM (0.1% versus 4.2%, $P < 0.0001$, t test) (Fig. 6B) (*31*, *32*). The CD34$^+$CD38$^-$ stem cell compartment in AA patients was more significantly depleted compared with the CD34$^+$CD38$^+$ progenitor compartment (Fig. 6A, left column, and Fig. 6, C and D). Moreover, HSCs and MPPs were virtually undetectable in the residual CD34$^+$CD38$^-$ compartment, confirming that HSCs are specifically lost in this clinical condition (Fig. 6A, column 2), at least at the phenotypic level. Despite the loss of phenotypic HSCs, the CD34$^+$CD38$^+$ compartment was detectable in all cases. We quantified the subsets within the CD34$^+$CD38$^+$ compartment to determine whether all cell types are indiscriminately affected. Based on our single-cell functional readouts, we grouped progenitors enriched for myeloid (CMP F1, MEP F1, and GMP) and erythroid (CMP F2/F3 and MEP F2/F3) differentiation potential to increase the power of the analysis due to relative loss of CD34$^+$ cells in AA. Despite significant depletion of HSCs, the percentage of myeloid progenitors was stable compared with normal BM (Fig. 6E). In contrast, erythroid progenitors were significantly lost, like HSCs, in all three patients analyzed (Fig. 6F, $P < 0.0001$, t test). These results suggest that ongoing erythropoiesis is more reliant on HSC input compared with myelopoiesis. Although we cannot rule out a specific Er lineage defect in AA, the pan-lineage deficiencies observed in AA likely rule out this possibility and support the idea that the Er progenitor loss is most likely a repercussion of HSC depletion. Because all HSC types and MPPs seem to be broadly lost in AA, our results cannot distinguish which type of HSC is crucial to maintain erythropoiesis. Recognizing that hematopoiesis in AA is not normal, the revised hierarchy model predicted from our experimental data does appear to have physiological relevance in the human setting.

Discussion

Our study challenges the current view that human blood development occurs progressively through a series of multipotent, oligopotent, and then unilineage progenitor stages. By subjecting the classically defined progenitor subsets to a sorting scheme that efficiently resolved My, Er, and Mk lineage fates, combined with single-cell functional analysis, we made two findings. First, we found that the cellular hierarchy of human blood is not identical across development. In FL, oligopotent progenitors with My-Er-Mk and Er-Mk activity were a prominent component of the hierarchy. By contrast, the BM was dominated by unilineage progenitors with primarily My or Er potential. This shift in progenitor classes demarcates a fundamental readjustment in the blood hierarchy during in utero to adulthood time points. The absence of oligopotent intermediates that become gradually restricted to unilineage progenitors in BM cannot be reconciled under the standard model of blood differentiation. Instead, our data support a hierarchy composed mainly of two tiers in adults: a top tier that contains multipotent cells such as HSCs and MPPs, and a bottom tier composed of committed unipotent progenitors (Fig. 7). We cannot formally rule out the presence of a highly transient adult CMP-like progenitor stage that exists when multipotent cells differentiate into unipotent progenitors; however, our in vitro and in vivo assays surveyed large cell numbers (~3000), and such a progenitor was not detected. If lineage-restricted cell types able to generate a subset or full spectrum of myeloid cells do exist in the stem cell compartment of adult marrow, they most probably represent the murine counterparts of myeloid-biased or myeloid-restricted HSC subtypes (*7*, *34*). Second, we found the origins

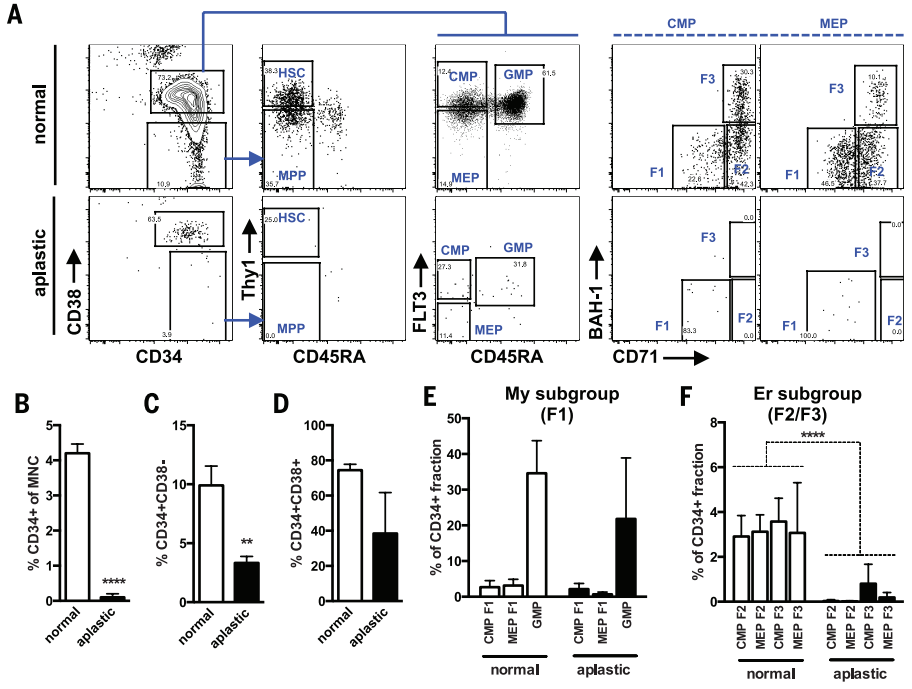

Fig. 6. Analysis of My and Er progenitors in patients with AA. (**A**) To examine the consequences of HSC loss on progenitor subsets, BM cells from three AA patients and normal controls were subjected to the new sorting design shown in Fig. 1B. Representative flow plots from a single AA case and a control are shown. (**B**) Quantification of total CD34$^+$ cells as a fraction of MNC pool from controls (empty bars) versus AA (filled bars). (**C** and **D**) The CD34$^+$ subset from controls and AA was further subdivided into the stem (CD34$^+$CD38$^-$) and progenitor (CD34$^+$CD38$^+$) cell compartments. (**E**) Analysis of My-enriched subsets (CMP F1, MEP F1, and GMP) in controls and in AA. (**F**) Analysis of Er-enriched subsets (CMP F2/F3 and MEP F2/F3) in controls and in AA. Bars indicate mean ± SE from three controls and three cases of AA. Asterisks indicate significance based on t test (**$P < 0.01$, ****$P < 0.0001$).

of the Mk lineage branch change from FL to BM. In FL, Mk progenitors were enriched but not restricted to the stem cell compartment, whereas in BM, the Mk lineage was closely tied to the fate of multipotent cells. These data are not consistent with the principal tenet of the standard model that My, Er, and Mk lineages originate from a common lineage progenitor such as CMP.

Historically, the first major lineage bifurcation step in the blood hierarchy was considered to be the segregation of myeloid (My-Er-Mk) and lymphoid fates (B, T, or Nk), with CMPs occupying the lineage fork that gives rise to the entire myeloid arm. The coemergence of My, Er, and Mk lineages was central to the description of CMPs. Our results reveal that the originally defined CMPs are highly heterogeneous, primarily composed of unipotent My or Er progenitors, with little Mk activity. In the absence of CMPs, how can the origins of lineage-restricted progenitors such as GMPs and MEPs be reconciled? Our previous clonal analysis suggested that myelomonocytic lineages originate from MLPs, which we suspect are the most probable precursor of GMPs, owing to their shared functional and transcriptional profiles (*11*, *12*, *35*). In this study, we found that *GATA-1*–positive Mk-Er–committed progenitors exist in the stem cell compartment, suggesting that MEPs are derived from multipotent cells. In the murine bone marrow niche, up to a quarter of LT-HSCs lie directly adjacent to Mks (*36*–*38*). Mks play a dual role in HSC regulation. Under normal conditions, Mk-HSC contact is essential to preserve the quiescent nature of adult LT-HSCs. After myeloablation, this effect is temporarily abrogated and Mks secrete growth factors that permit HSCs to expand (*37*). Thus, direct differentiation of Mks from HSCs may represent a physical mechanism to regulate blood stem cell functionality in the niche. Overall, the first major bifurcation step in blood differentiation is far more complex than a simple segregation of myeloid and lymphoid lineages. In humans, we suspect that this first step splits the Mk-Er lineage from the myelomonocytic lineage that cosegregates with the lymphoid fate (*11*). Why the myelomonocytic lineage, but not the granulocytic lineage, is tied to the lymphoid fate will be a critical area of future investigation (*39*, *40*). The ability to isolate developmental populations reported here provides a critical experimental framework to facilitate such studies in humans.

Our data may also have implications for our understanding of lineage specification at the molecular level. *vWF*, a key molecular marker strongly associated with Mk differentiation, was expressed in a subset of BM HSCs and may identify an HSC subtype primed for platelet production as shown in mouse (*16*). Molecular factors involved in Mk (*vWF*), Er (*EPOR*), and My (*CSF2RA*) differentiation were expressed in small pockets of single cells from undifferentiated cells, like BM HSCs, in a near mutually exclusive manner. This is consistent with the notion that transcription factors (TFs) associated with myeloid lineage specification are individually but not simultaneously primed at the level of stem cells. It is difficult to preclude that this type of molecular heterogeneity reflects impurities in human HSC isolation. However, analysis of purer murine HSC compartments supports that molecular factors associated with lineage commitment are stochastically activated at the level of HSCs (*16*, *17*). Maintenance of a multipotent state is thought to occur via low-level lineage priming, where TFs of different lineages are coexpressed in the same cell. In this model, commitment toward a particular lineage occurs by the mutual antagonism of these TFs, where one TF eventually wins, locking in a differentiation program. However, the near mutually exclusive expression of *vWF*, *CSFR2A*, and *EPOR* in BM HSCs does not agree with this logic. The hypothesis that lineage commitment occurs in the absence of a coordinated differentiation program (*17*, *25*, *41*) is more consistent with our data.

Ultimately, a better understanding of normal blood differentiation programs will be critical to deciphering how such programs go awry in disease. Indeed, in AA, we observed that Mk-Er progenitors are lost alongside HSC, but the My progenitor pool can continue to persist. Recent evidence from murine in situ tracking expriments showed that My progenitors can be sustained long term without contribution from HSCs (*27*, *28*). If human My progenitors are similarly long-lived, they would have a higher probability of acquiring mutations that could lead to clonal expansion and eventual My lineage leukemias. The short-lived nature of Mk-Er progenitors and their dependency on HSC input reduce their probability of accumulating enough mutations leading to leukemia. Clinical evidence that acute leukemia of the Mk and Er lineages is extremely rare compared with My leukemia is consistent with this idea. Our work on AA highlights one example of the clinical utility of a high-resolution developmental road map of normal hematopoiesis. The adaptable nature of our new sorting scheme should similarly inform on other hematological conditions.

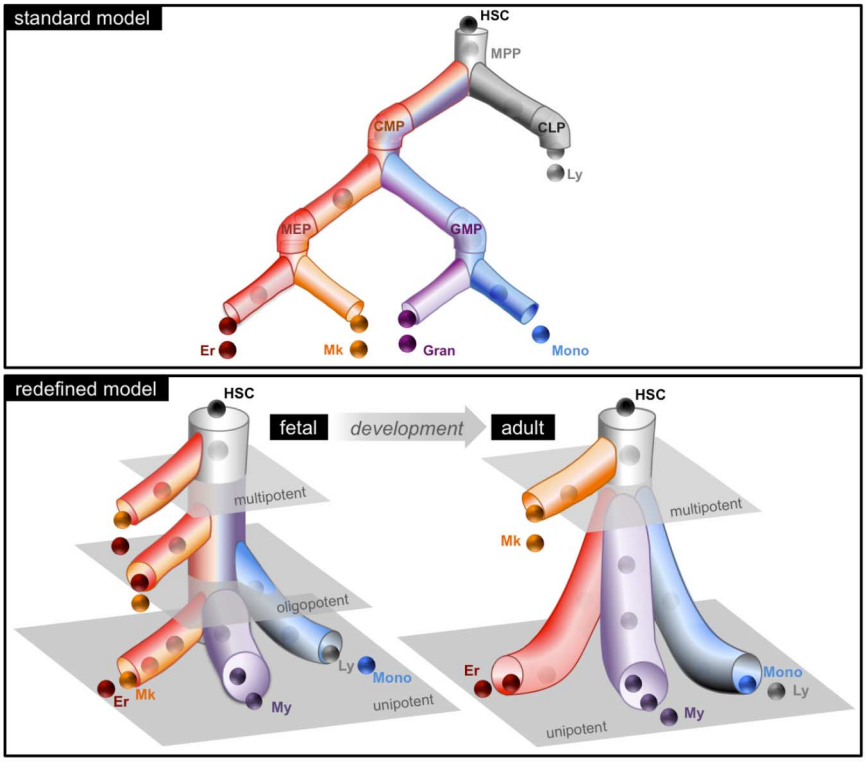

Fig. 7. A model of the changes in human My-Er-Mk differentiation that occur across developmental time points. Graphical depiction of My-Er-Mk cell differentiation that encompasses the predominant lineage potential of progenitor subsets; the standard model is shown for comparison. The redefined model proposes a developmental shift in the progenitor cell architecture from the fetus, where many stem and progenitor cell types are multipotent, to the adult, where the stem cell compartment is multipotent but the progenitors are unipotent. The grayed planes represent theoretical tiers of differentiation.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/351/6269/aab2116/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S7
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Editor's Summary

Adjusting hematopoietic hierarchy

In adults, more than 300 billion blood cells are replenished daily. This output arises from a cellular hierarchy where stem cells differentiate into a series of multilineage progenitors, culminating in unilineage progenitors that generate over 10 different mature blood cell types. Notta *et al.* mapped the lineage potential of nearly 3000 single cells from 33 different cell populations of stem and progenitor cells from fetal liver, cord blood, and adult bone marrow (see the Perspective by Cabezas-Wallscheid and Trumpp). Prenatally, stem cell and progenitor populations were multilineage with few unilineage progenitors. In adults, multilineage cell potential was only seen in stem cell populations.

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