PLATELETS AND THROMBOPOIESIS

A lineage of diploid platelet-forming cells precedes polyploid megakaryocyte formation in the mouse embryo

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Key Points

• The first embryonic platelets are produced by a unique lineage of diploid cells not polyploid MKs.
• Diploid platelet-forming cells are produced in the early mouse embryo via a progenitor cell–independent pathway.

Introduction

Blood cell formation first occurs within the embryonic yolk sac (YS), yielding primitive red cells and multiple classes of colony-forming units (CFUs)/hematopoietic progenitor cells (HPCs).1 From embryonic day (E) 7.5, the YS contains megakaryocyte (MK) CFUs2–4 and, by E8.5, is capable of generating polyploid MKs in vitro.2 Detection of circulating platelets soon follows the appearance of HPCs.4 The intuitive extrapolation is that MK-CFUs differentiate within the YS to initiate platelet formation, but this model remains untested. To better understand how platelet production proceeds in the YS, we have employed a combination of functional assays, transcriptomics, imaging, and gene disruption to investigate the relationship between HPCs and generation of the first platelet-forming cells.

Study design

UBI-gfp,5 Runx1LMac2LacZ,6 and Runx1−/− mouse lines were maintained as C57BL/6. Walter and Eliza Hall Institute Animal Ethics Committee approved the experiments. Developmental stages were determined morphologically or by counting somite pairs.

For CFU-MK culture, MegaCult-C (StemCell Technologies) was used as previously described;2 colonies were defined as clusters of ≥10 CD41 cells after 5 days. M3434 (StemCell Technologies) was used for myelo-erythroid assays.

Proplatelet assays were performed using serum-free medium.9 Time-lapse imaging was performed using microgrid arrays (Microsurfaces) attached to coverslip chamber slides.10

An LSM 780 microscope was used for confocal imaging. Analysis was performed using Imaris (Bitplane).

For transcriptome analyses, samples were hybridized to Illumina Expression BeadChips (ArrayExpress accession: E-MTAB-2625) and analyzed using limma.

Results and discussion

Although MK-CFUs have been functionally identified in the YS,2,4 the immunophenotype of these progenitors is undescribed, making it unclear how they relate to the conventional CD45−CD41low HPC population.11,12 To address this, we isolated cells expressing CD45 and/or CD41 from E9.5 and E10.5 YS (Figure 1A) and performed in vitro colony-forming assays. At both stages, all MK-CFUs present in whole YS were accounted for by the conventional HPC population (Figure 1B; supplemental Figure 1, see the Blood Web site).

We found that platelets first entered the peripheral blood (PB) from E9.5, increasing rapidly in number by E10.5 (Figure 1C). It would therefore be expected that the cells responsible for platelet formation first occur within the embryonic yolk sac (YS), which are endowed with megakaryocytic potential, differentiate into the first platelet-forming cells in vivo. We demonstrate that from embryonic day (E) 8.5 all megakaryocyte (MK) colony-forming cells belong to the conventional hematopoietic progenitor cell (HPC) compartment. Although these cells are indeed capable of generating polyploid MKs, they are not the source of the first platelet-forming cells. We show that proplatelet formation first occurs in a unique and previously unrecognized lineage of diploid platelet-forming cells, which develop within the YS in parallel to HPCs but can be specified in the E8.5 Runx1−null embryo despite the absence of the progenitor cell lineage. (Blood. 2014;124(17):2725-2729)

In this study, we test the assumption that the hematopoietic progenitor/colony-forming cells of the embryonic yolk sac (YS), which are endowed with megakaryocytic potential, differentiate into the first platelet-forming cells in vivo. We demonstrate that from embryonic day (E) 8.5 all megakaryocyte (MK) colony-forming cells belong to the conventional hematopoietic progenitor cell (HPC) compartment. Although these cells are indeed capable of generating polyploid MKs, they are not the source of the first platelet-forming cells. We show that proplatelet formation first occurs in a unique and previously unrecognized lineage of diploid platelet-forming cells, which develop within the YS in parallel to HPCs but can be specified in the E8.5 Runx1−null embryo despite the absence of the progenitor cell lineage. (Blood. 2014;124(17):2725-2729)
Figure 1. Identification of diploid platelet-forming cells (DPFCs) in the E10.5 YS. (A) CD45 and CD41 expression in pooled E10.5 YS. Values indicate the numbers (mean ± standard error of the mean [SEM]) of each population per embryo equivalent (n = 15). (B) Distribution of CFUs with MK potential in 1 embryo equivalent of whole or purified E9.5 and E10.5 YSs. Cells were dissociated in 10% collagenase/dispase for 45 minutes at 37°C and then dissociated mechanically. No CD45−CD41+ cells were present in E9.5 YS. Error bars represent SEM (n = 3). sp, somite pairs. (C) To distinguish embryonic from contaminating maternal platelets in preparations of embryonic peripheral blood, green fluorescent protein (GFP)–expressing male mice were mated with wild-type females, ensuring that all GFP+ cells were of embryonic origin. Flow cytometry plots showing the presence of GFP+ platelets in the PB of E9.5 (i) and E10.5 (ii) embryos. (iii) Quantification of embryonic platelets in the PB of E9.5–10.5 embryos. Error bars represent standard deviation (SD) (n = 9–21 embryos per stage). (D) (i) Representative image of E10.5 YS CD45−CD41+ HPCs cultured in proplatelet medium (including anti-CD41-APC) for 72 hours (n = 5). Red indicates CD41 expression. Scale bar represents 40 μm. (ii) DNA content analysis of HPC-derived CD41highCD42D− cells demonstrating a conventional MK 2–16n ploidy profile (n = 3). Following antibody staining, cells were fixed in 80% ethanol, permeabilized in TritonX-100, and stained with 4,6-diamidino-2-phenylindole (DAPI). (E) Multidimensional scaling plot of microarray data comparing transcriptional similarity of E10.5 YS CD45−CD41− cells with
production are terminally differentiated by E10.5. Hallmarks of mature MKs are their highly polyploid nuclei and the ability to form proplatelets.\textsuperscript{13} Proplatelets can be acutely induced ex vivo from MKs within hours,\textsuperscript{14} whereas production of proplatelet-forming MKs from fetal precursors require at least 4 days.\textsuperscript{15} We found that after 72 hours in vitro HPCs generated polyploid MKs, but proplatelets were rarely formed (Figure 1D), indicating that HPCs had not differentiated into acute proplatelet-forming cells in vivo and were therefore not the source of the first platelets.

We next queried if an alternative population was responsible for platelet formation. By comparing the transcriptional profiles of E10.5 YS cells with E13.5 liver reference lineages, including MKs, we investigated if elements of an MK signature were present at E10.5. We found that a previously uncharacterized population of CD45\textsuperscript{+} CD41\textsuperscript{high} cells was strikingly similar to E13.5 MKs (Figure 1E, supplemental Figure 2A); this population also coexpressed MK-associated proteins including MPL (Myeloproliferative leukemia virus oncogene), CD42D, and acetylcholinesterase (supplemental Figure 2B-C). Curiously, CD45\textsuperscript{+} CD41\textsuperscript{high} cells did not exhibit the high ploidy range associated with conventional MKs or E10.5 YS HPC-derived MKs; rather, the majority were diploid (Figure 1F).

Approximately half of the low-ploidy CD45\textsuperscript{+} CD41\textsuperscript{high} cells acutely produced proplatelets in vitro (Figure 1G). Similarly to FL MKs,\textsuperscript{16} in vitro thrombopoiesis was not THPO dependent (supplemental Figure 2D), but surprisingly, proplatelets were formed while in a diploid state (Figure 1H).

Using a refined MK immunophenotype (CD41\textsuperscript{high}CD42D\textsuperscript{+}), we confirmed that proplatelet formation also occurred in a diploid state in vivo (Figure 1I-J). We therefore defined this lineage as DPFCs.

That HPC-derived MKs were highly polyploid yet in vivo platelet-forming cells were diploid prompted us to ask whether HPCs are the source of YS DPFCs in vivo. To address this, we deconjugated this lineage as DPFCs.

CD41\textsuperscript{high}CD42D\textsuperscript{+} cells were generated in vitro from E7.5 and E7.75 YSs, but acute proplatelet formation was rarely observed, and only from E7.75 (Figure 2A). By E8.5 CD41\textsuperscript{low/−}TER119\textsuperscript{−} primitive erythroid (EryP)\textsuperscript{11} and VECAD\textsuperscript{−} CD41\textsuperscript{high} HPC lineages can be prospectively isolated.\textsuperscript{11,17} Accompanying these, we identified a population of VECAD\textsuperscript{−} CD41\textsuperscript{high} cells that encompassed all CD42D\textsuperscript{+} cells (Figure 2B), indicating that they might include acute proplatelet-forming cells.

CFU and platelet assays revealed that VECAD\textsuperscript{−} CD41\textsuperscript{high} cells contained myeloid/erythroid and MK CFUs, but only VECAD\textsuperscript{−} CD41\textsuperscript{high} cells were capable of acute proplatelet formation (Figure 2C-D, supplemental Figure 3). That E8.5 VECAD\textsuperscript{−} CD41\textsuperscript{high} cells displayed little acetylcholinesterase activity (supplemental Figure 4) suggested that they are an immature DPFC precursor. Thus, as early as E8.5, acute proplatelet-forming cells exist in the YS and are immunophenotypically distinct from the HPC lineage.

Hematopoietic commitment in the YS occurs via VECAD-expressing precursors.\textsuperscript{18,19} Consistent with this, all CD41-expressing cells in the E7.75–8.25 blood band coexpressed VECAD, but by E8.5, they had diverged into VECAD\textsuperscript{+} (HPC) and VECAD\textsuperscript{−} (pre-DPFC) counterparts (supplemental Figure 5). This suggested HPC and DPFC lineages both progressed via VECAD-expressing precursors. We questioned whether YS DPFCs arise in parallel to, but independently of, HPCs.

Determining whether a lineage is HPC derived in the YS has been problematic. The best attempts have used c-Myb\textsuperscript{−/−} mice.\textsuperscript{20} However, multiple classes of HPCs are produced in c-Myb\textsuperscript{−/−} embryos; among these are erythroid, macrophage, and MK CFUs.\textsuperscript{21,22} We reasoned that a more stringent approach would be the use of Runx1-null embryos. Without RUNX1, HPC formation is completely blocked\textsuperscript{22,23}; yet hematopoietic specification from the mesoderm is permitted, as evidenced by EryP formation.\textsuperscript{24} Thus, studying the Runx1-null YS should allow us to test if HPCs give rise to DPFCs.

Using 2 independent Runx1-null lines,\textsuperscript{6,7} (both carrying the null allele throughout the germ line), we found that the E8.5 Runx1-null YSs contained VECAD\textsuperscript{−} CD41\textsuperscript{high} pre-DPFCs and were capable of generating CD41\textsuperscript{−} CD42D\textsuperscript{+} cells in vitro (Figure 2E-F). Analysis of E10.5 Runx1-null YS confirmation that the absence of HPCs, platelet-producing DPFCs were formed in vivo (Figure 2G-H). Although Runx1-null DPFC numbers were lower both in vitro and in vivo, which is likely a result of RUNX1 being an essential factor for megakaryopoiesis,\textsuperscript{25} these proof-of-concept experiments demonstrated that developmental specification of the DPFC lineage was not HPC dependent.

In summary, we have shown that polyploid MKs generated from YS cultures\textsuperscript{2} which resemble MKs of fetal life, are the product of HPC differentiation. These do not represent the first in vivo platelet-forming cells of the embryo. Platelet formation in the YS is initiated by a previously unrecognized cell that we have termed DPFC, which likely develops via the primitive hematopoietic pathway.\textsuperscript{2,4} A key challenge will now be to experimentally define when HPC-derived conventional MKs supersede DPFCs to become the source of platelets during fetal life.
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Authorship


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