PLATELETS AND THROMBOPOIESIS

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

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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)

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). From embryonic day (E) 7.5, the YS contains megakaryocyte (MK) CFUs and, by E8.5, is capable of generating polyploid MKs in vitro. Detection of circulating platelets soon follows the appearance of HPCs. 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 Runx1LacZ/LacZ, and Runx1T3/17 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; 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


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There is an Inside Blood Commentary on this article in this issue.

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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 CD41^+CD42D^1 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.

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 lines, 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{hi}CD42D\textsuperscript{−} 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{hi}CD42D\textsuperscript{−} 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{hi}CD42D\textsuperscript{−} 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{hi}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 investigated when DPFC commitment first occurred.

CD41\textsuperscript{hi}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{11,17} population of VECAD\textsuperscript{11,17} cells were generated in vitro from E7.5 and E7.75 YSs that are in the process of platelet production in vivo. Surface objects for nuclei were stained with DAPI. Colors reflect the intensity sum of DAPI signal, ranging from the 2n (blue) to 4n (red) states. Scale bar represents 10 μm.

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Authorship


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References


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