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 megakaryocytes.
- Diploid platelet-forming cells are produced in the early mouse embryo via a progenitor cell-independent pathway.

Abstract

In this study we test the assumption that the hematopoietic progenitor/colony-forming cells of the embryonic yolk sac, which are endowed with megakaryocytic potential, differentiate into the first platelet-forming cells \textit{in vivo}. We demonstrate that from embryonic day (E) 8.5 all megakaryocyte-colony forming cells belong to the conventional hematopoietic progenitor cell compartment. Although these cells are indeed capable of generating polyploid megakaryocytes, 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 yolk sac in parallel to hematopoietic progenitor cells but can be specified in the E8.5 \textit{Runx1}-null embryo despite the absence of the progenitor cell lineage.
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)-CFUs \(^2-4\) and by E8.5 is capable of generating polyploid megakaryocytes \textit{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.

Methods

UBI-gfp \(^5\), Runx1\(^\text{LacZ/LacZ}\) \(^6\), and Runx1\(^\Delta/\Delta\) \(^7\) mouse lines were maintained as C57BL/6. WEHI Animal Ethics Committee approved experiments. Developmental stages were determined morphologically or by counting somite pairs.

For CFU-MK culture MegaCult-C (StemCell Technologies) was used as previously described \(^8\); colonies were defined as clusters of \(\geq 10\) CD41\(^+\) cells after five days. M3434 (StemCell Technologies) was used for myeloid-erythroid assays.
Proplatelet assays were performed using serum-free medium. Time-lapse imaging was performed using microgrid arrays (Microsurfaces) attached to coverslip chamber slides.

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, the immunophenotype of these progenitors is undescribed, making it unclear how they relate to the conventional CD45^CD41^low HPC population. 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, Figure S1).

We found that platelets first entered the peripheral blood from E9.5, increasing rapidly in number by E10.5 (Figure 1C). It would therefore be expected that the cells responsible for platelet production are terminally differentiated by E10.5. Hallmarks of mature MKs are their highly polyploid nuclei and the ability to form proplatelets. Proplatelets can be acutely induced ex vivo from MKs within hours, while production of proplatelet-forming MKs from fetal precursors require at least four days. We found that after 72hrs 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 to E13.5 liver reference lineages, including MKs, we investigated if elements of a MK signature were present at E10.5. We found that a previously uncharacterized population of CD45$^-$CD41$^{\text{high}}$ cells was strikingly similar to E13.5 MKs (Figure 1E, Figure S2A); this population also co-expressed MK-associated proteins including MPL, CD42D, and acetylcholinesterase (Figure S2B-C). Curiously, CD45$^-$CD41$^{\text{high}}$ 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$^-$CD41$^{\text{high}}$ cells acutely produced proplatelets in vitro (Figure 1G). Similarly to fetal liver MKs in vitro thrombopoiesis was not THPO dependent (Figure S2D), but surprisingly proplatelets were formed while in a diploid state (Figure 1H).

Using a refined MK immunophenotype (CD41$^{\text{high}}$CD42D$^+$) we confirmed that proplatelet formation also occurred in a diploid state in vivo (Figures 1I-J). We therefore defined this lineage as diploid platelet-forming cells (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{high}CD42D\textsuperscript{+} cells were generated \textit{in vitro} from E7.5 and E7.75 YS, 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 VECADHERIN\textsuperscript{+}(VECAD)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 proplatelet assays revealed that VECAD\textsuperscript{+}CD41\textsuperscript{high} cells contained all Myeloid/Erythroid and MK CFUs, but only VECAD\textsuperscript{-}CD41\textsuperscript{high} cells were capable of acute proplatelet formation (Figure 2C-D, Figure S3). That E8.5 VECAD\textsuperscript{-}CD41\textsuperscript{high} cells displayed little acetylcholinesterase activity (Figure S4) 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 co-expressed VECAD, but by E8.5 had diverged into VECAD\textsuperscript{+} (HPC) and VECAD\textsuperscript{-} (pre-DPFC) counterparts (Figure S5). This suggested that 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. Best attempts have utilized \textit{c-Myb}\textsuperscript{−/−} mice \textsuperscript{3,20}. However, multiple classes of HPC are produced in \textit{c-Myb}\textsuperscript{−/−} embryos, amongst these are erythroid (CFU-E), macrophage, and MK CFUs \textsuperscript{3,21}. We reasoned that a more
stringent approach would be the use of Runx1-null embryos. Without RUNX1 HPC formation is completely blocked \(^{22,23}\), yet hematopoietic specification from the mesoderm is permitted, as evidenced by EryP formation \(^{24}\). Thus, studying the Runx1-null YS should allow us to test if HPCs give rise to DPFCs.

Using two independent Runx1-null lines \(^{6,7}\) (both carrying the null allele through the germline), we found that the E8.5 Runx1-null YS contained VECAD^CD41\(^{\text{high}}\) pre-DPFCs and were capable of generating CD41^CD42D^ cells \textit{in vitro} (Figure 2E,F). Analysis of E10.5 Runx1-null YS confirmed that despite the absence of HPCs platelet-producing DPFCs were formed \textit{in vivo} (Figure 2G,H). Although Runx1-null DPFC numbers were lower both \textit{in vitro} and \textit{in vivo}, which is likely a result of RUNX1 being an essential factor for megakaryopoiesis \(^{25}\), 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 \(^2\), which resemble MKs of fetal life, are the product of HPC differentiation. These do not represent the first \textit{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 develop via the primitive hematopoietic pathway \(^2,4\). 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 contribution

KP, ST, WSA, and DJH designed the research, analyzed data, and wrote the paper. JFM, CB, ECJ, LWW, KR, AL, BTK, and AM designed the research. TS, WS, and GKS analyzed data.

The authors declare no competing financial interests.
References


Figure 1. Identification of diploid platelet-forming cells in the E10.5 yolk sac.

(A) CD45 and CD41 expression in pooled E10.5 YS. Values indicate the numbers (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 YS. Cells were dissociated in 10% collagenase/disparse for 45 mins at 37°C and then dissociated mechanically. No CD45⁺CD41⁻ cells were present in E9.5 YS. Error bars = SEM (n=3). SP, somite pairs.

(C) To distinguish embryonic from contaminating maternal platelets in preparations of embryonic peripheral blood (PB), GFP-expressing male mice were mated with wildtype females, ensuring that all GFP+ cells were of embryonic origin. (i-ii) 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, SD. (n=9-21 embryos per stage)

(D) (i) Representative image of E10.5 YS CD45⁺CD41³low HPCs cultured in proplatelet medium (including anti-CD41-APC) for 72hrs (n=5). Red = CD41 expression. Scale bar, 40μm. (ii) DNA content analysis of HPC-derived CD41³highCD42D⁺ cells demonstrating a conventional megakaryocyte 2-16n ploidy profile (n=3). Following antibody staining, cell were fixed in 80% ethanol, permiabilized in TritonX-100, and stained with DAPI.

(E) Multidimensional scaling plot of microarray data comparing transcriptional similarity of E10.5 YS CD45⁺CD41³high cells to: E10.5 YS lineages (HPC = CD45⁺CD41³low, myeloid = CD45⁺CD41⁻, endothelial = VECAD⁺CD45⁺CD41⁻); and E13.5 fetal liver (FL) lineages (blast = CD45⁺CD11B⁻, myeloid = CD45⁺CD11B⁺, and megakaryocyte = CD41³highCD42D⁺CD150⁺). Note that myeloid and HPC populations from the YS cluster with their FL counterparts, while YS CD45⁺CD41³high cells cluster most strongly with E13.5 FL megakaryocytes.

(F) DNA content analysis of E10.5 YS CD45⁺CD41³high cells demonstrating that these cells are predominantly 2n in vivo (n=3).

(G) Cumulative frequency plot of in vitro proplatelet forming capacity of 145 CD45⁺CD41³high pedigrees. Cells were cultured in serum-free medium with
100ng/ml recombinant mouse THPO (including anti-CD41-APC) and live-imaged at a 3-4 minute time resolution. Data from three independent experiments are shown. For each experiment, 6000 cells were plated per chamber. The number of microwells imaged was limited by the number of positions that could be imaged with a 3-4 minute time period. n = number of pedigrees per experiment.

(H) Representative confocal z-stack of E10.5 YS CD45^CD41^{high} cells after 72 hours in serum-free medium with 100ng/ml recombinant mouse THPO (n=30). Cultures were fixed in 2% PFA for 20 minutes, permeabilized with 0.6% TritonX/10% FCS, and stained with anti-CD41 and anti-CD42D antibodies (0.3% TritonX/5% FCS), nuclei were stained with DAPI. Note that cells are generating proplatelets while in a diploid state. Scale bar, 10μm.

(I) Scatter plot of object volumes and relative DNA content of CD41^{high}CD42D^+ cells within E10.5 yolk sacs that are in the process of platelet production in vivo. Surface objects for nuclei were generated by manually defining the area of DAPI staining within individual cells using Imaris software (Bitplane); intensity sum values representing the 4n state were generated from mitotic figures from CD41^CD42D^- cells within the z-stack. Relative DNA content was derived by dividing the intensity sum of CD41^{high}CD42D^+ nuclei by that of a mitotic figure within the same z-stack. This strategy allows determination of ploidy of cells within the z-stack that are, or are not, in the process of platelet production. A value of 0.5 represents 2n and 1.0 represents 4n. Data are derived from 10 individual z-stacks taken from 5 yolk sacs.

(J) Representative confocal z-stack of CD41^{high}CD42D^+ cells within the VECAD-expressing vasculature of the E10.5 yolk sac in vivo. Surface objects of DNA (DAPI) content are overlaid, and reflect the range of DNA content of CD41^{high}CD42D^+ cells in vivo. The diploid state of a proplatelet forming cell (blue arrowhead) is highlighted. Freshly dissected yolk sacs were fixed in 2% PFA for 20 minutes, permeabilized with 0.6% TritonX/10% FCS for 30 minutes, and stained with anti-CD41, -VECAD, and -CD42D antibodies (0.3% TritonX/5% FCS), nuclei were stained with DAPI. Colours reflect the intensity sum of DAPI signal, ranging from the 2n (blue) to 4n (red) states. Scale bar, 10μm; n=20.
Figure 2. Diploid platelet-forming cells emerge in parallel to the progenitor cell lineage.

(A) Representative image of E7.5 and E7.75 YS cultures after 72hrs. Scale bar, 20μm. After dissection, YSs were incubated in 0.25% trypsin/EDTA at 37°C for 5 minutes, mechanically dissociated, and then cultured in serum-free medium with 100ng/ml recombinant mouse THPO (anti-CD41-APC and -CD42D-PE antibodies were added to the culture medium). Note that CD41^+CD42D^+ cells (arrows) were generated from both stages but proplatelets (arrow head) were only from E7.75 cultures. (n=3). Scale bar, 30μm.

(B) Plots of VECAD, CD41, CD42D and TER119 expression in pooled E8.5 yolk sacs (n=3). SP, somite pairs.

(C) Distribution of E8.5 CFU-EryP (white), -Myeloid/Erythroid (grey) and -MK (black) in 1 embryo equivalent of sorted YS cells. Error bars = SEM (n=3). EryP colonies were distinguished from BFU-E (which contain both adult-type red cells and one leucocyte lineage) according to morphology.

(D) Representative images from cultures of purified E8.5 YS populations cultured in proplatelet medium (with anti-CD41-APC and anti-CD42D-PE) for 72hrs (n=3). Scale bar, 30μm. Although VECAD^CD41^high cells generated CD41^+CD42D^+ megakaryocytes, proplatelet-forming CD41^+CD42D^+ cells (arrow) were only observed from cultures of VECAD^CD41^high cells.

(E) Representative plots from Runx1^+/+ (n=5) and Runx1^Δ/Δ (n=6) E8.5 YS cells at the 8-somite pair (sp) stage demonstrating that the VECAD^CD41^high proplatelet-forming lineage is specified in the absence of RUNX1. Values indicate the numbers of cells detected (mean±SD).

(F) E8.5 Runx1^Δ/Δ YSs were capable of forming CD41^+CD42D^+ cells after 72 hrs in the proplatelet assay (i), these cells are capable of proplatelet formation in vitro (ii). Values represent the mean ± SEM. (n=3). Scale bar, 30 μm.

(G) Representative plot of CD45 and CD41 expression in E10.5 YS from Runx1^+/+ (n=6) and Runx1-null (n=8) embryos demonstrating that in the absence of RUNX1 DPFCs can develop (red gate) while HPCs are completely absent (blue gate). Values indicate the numbers of cells detected (mean±SD).
Representative (n = 6) confocal z-stack of $Runx1^{Δ/Δ}$ E10.5 YS showing CD41$^{\text{high}}$CD42$^+$ DPFCs and platelets (arrow) can be produced in vivo despite the complete absence of HPCs. Bar, 10μm. Inset, optical section of boxed region showing a free platelet (arrow) within the $Runx1$-null yolk sac. Bar, 5μm.
A lineage of diploid platelet-forming cells precedes polyploid megakaryocyte formation in the mouse embryo