adhesion.[13] Interestingly, these mice do not present any obvious defects in tissues where podocalyxin and CD34 are coexpressed (hematopoietic and vascular cells), suggesting functional compensation between the CD34-related molecules. Consistent with this hypothesis, we have observed up-regulated expression of CD34 in *podxl*$^{-/-}$ tissues.[13]

To better understand podocalyxin's role in embryogenesis, we have now performed a detailed analysis of its expression in murine development with particular attention to hematopoietic tissues. Our data suggest that podocalyxin is expressed by the earliest hematopoietic progenitors (hematopoietic stem cells [HSCs]) and by erythroid precursors during embryonic development but that its expression on erythroid lineage cells ceases shortly after birth. Interestingly, erythroid expression of podocalyxin is rapidly reactivated in the adult in response to hemolytic anemia, suggesting its expression by these cells may reflect an erythropoietic stress response. On normal adult BM, we find that podocalyxin expression is restricted to a rare population of cells bearing a stem cell phenotype (lineage marker$^-$ [Lin$^-$] Sca-1$^+$ c-kit$^+$ [LSK]). This was confirmed in transplantation experiments: Podocalyxin-positive LSK cells were capable of serially transplanting myeloid and lymphoid lineages in lethally irradiated mice. In summary, our data suggest that podocalyxin is indeed a highly restricted marker of long-term repopulating HSC in adult BM and that it is present on these cells throughout development. In light of its role in blocking cell adhesion in vitro and its prominent expression by hematopoietic precursors when hematopoietic tissues are actively seeded, we propose that podocalyxin may act as an invasive factor to make these cells more mobile and aid in the seeding of new hematopoietic compartments.

Materials and methods

Mice

Wild-type (*wt*), Ly5.1–enhanced green fluorescent protein (Ly5.1-EGFP), *podxl*$^{+/-}$, *cd34*$^{-/-}$, and *podxl*$^{+/-}$*cd34*$^{-/-}$ mouse strains (all in a C57BL/6 background) were bred and maintained at The Biomedical Research Centre (BRC), University of British Columbia (UBC). Gestational age of embryos was determined by the date of copulation and morphologic landmarks as previously described.[21] Mice used in phenylhydrazine (PHZ) experiments were all more than 8 weeks old.

Cell preparation

Single-cell suspensions were prepared from YS, FTL, SPL, and BM by drawing cells through a 25-gauge needle and then expelling them through a 70 μm nylon mesh. Cells were suspended in either phosphate-buffered saline (PBS) containing 0.05% NaN_3 and 10% fetal calf serum (FCS) or in Hanks balanced salt solution (HBSS) without phenol red (Sigma Chemical, St Louis, MO), supplemented with 2% FCS.

Antibodies

The rat monoclonal antibody against podocalyxin (PCLP1) has been described previously.[14] The immunoglobulin G_1 (IgG_1) control (Cedarlane, Hornby, ON) was used at 5 μg/mL to confirm the specificity of PCLP1 antibody, and in some cases *podxl*$^{-/-}$ tissues were used to verify the absence of nonspecific background staining. All other antibodies were obtained from BD PharMingen (San Diego, CA) and used as recommended by the manufacturer: biotinylated antibodies to platelet endothelial cell adhesion molecule-1 (PECAM-1)/CD31 (vascular endothelia), Ter119 (erythroid cells), Gr1 (8C5 [granulocytes]), B220 (6B2 [B lineage cells]), Mac1 (M1/70 [myelomonocytic cells]), and CD34 (RAM34 [progenitor cells]) and phycoerythrin (PE)–conjugated antibodies to Sca-1 (E13 [progenitor cells]), c-kit (2B8 [progenitor cells]), CD71 (C2 [proliferating cells and erythroblasts]), and CD45 (panhematopoietic).

Immunohistochemistry

Immunoperoxidase staining of frozen embryo sections was performed essentially as described previously.[13] Antibodies were used at the following dilutions/concentrations: PCLP1 (1:1000 [14]), PECAM-1/CD31 (2.5 μg/mL [BD PharMingen]), and control IgG_1 (5 μg/mL). Secondary antibodies were used as recommended by the manufacturer (Vectastain-ABC kit; Vector Laboratories, Burlingame, CA). Peroxidase activity was detected with diaminobenzidine (0.5 mg/mL) and 0.03% H_2O_2 in H_2O followed by methyl green counterstain; coverslips were mounted with Permount (Fisher Scientific, Hampton, NH). An Olympus BH-2 microscope (10 ×/0.3 aperture Olympus objective), a Sensys1401E digital camera (Roper Scientific, Duluth, GA), and MetaVue Software (Universal Imaging, Downingtown, PA) were used for acquisition of images.

Flow cytometry and cell sorting

For single-, dual-, and triple-color immunofluorescence analyses, 10^6 primary cells were preincubated with 5% goat serum in PBS containing 10% FCS and stained for PCLP1 followed by goat antirat IgG Alexa-fluor 488–coupled antibody (Molecular Probes, Eugene, OR). After extensive washes and blocking in 2% rat serum, biotin-conjugated monoclonal antibodies followed by allophycocyanin (APC)–coupled streptavidin (BD PharMingen) and PE-conjugated monoclonal antibodies were added to the cells as described previously.[22] All flow cytometric analyses were performed using a FACSCalibur (Becton Dickinson, San Jose, CA). 7-Amino actinomycin (7AAD) and light scatter properties were used to eliminate dead cells. Analyses were performed using CellQuest software (Becton Dickinson). Sorting of PCLP1$^+$ cells was performed on a FACSVantage (Becton Dickinson). After 2 rounds of sorting, single cells were deposited in a 96-well flat-bottom culture plate using the CloneCyte device and software (Becton Dickinson). For in vivo injection, 10^4 sorted cells were collected and diluted to 1000 cells per 200 μL HBSS before injection into the tail vein of recipient mice for spleen colony-forming unit (CFU-S) assays.

Hematopoietic reconstitution

Cell sorting of PCLP1$^+$ cells was performed on a FACSVantage cell sorter (Becton Dickinson). After 2 rounds of sorting, 10 to 500 Ly5.1 LSK cells expressing high levels of PCLP1 antigen or LSK cells negative for this antigen were deposited in a 96-well flat-bottom culture plate using the CloneCyte device and software (Becton Dickinson). About 300 000 Sca-1–depleted Ly5.2-GFP helper BM cells were added to the donor cells before tail vein injection into lethally irradiated Ly5.2 recipients. Reconstituted mice were periodically bled via the tail vein to monitor reconstitution by donor-marked (Ly5.1$^+$) progenitors. Serial transplantations were performed after 4 months for animals presenting more than 10% BM chimerism. Total BM (10^6 cells) from the original recipients was reinjected into the tail vein of lethally irradiated Ly5.2 secondary and tertiary recipients. All flow cytometric analyses were performed using a FACSCalibur flow cytometer (Becton Dickinson). 7AAD-positive cells and cells with high obtuse scatter or nonphysiologically low forward scatter were excluded from all sorts and analyses to eliminate dead cells and debris.

Migration assays

FTL cells (E15) from *wt*, *podxl*$^{-/-}$, or *podxl*$^{-/-}$*cd34*$^{-/-}$ were either labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) at 5 μM per 10^7 cells per milliliter or carboxylic acid SNARF-1 acetate succinimidyl ester (CSA-SE) at 10 μM per 10^7 cells per milliliter (Molecular Probes) as indicated; the cells were then washed and mixed at an equal ratio before transplantation. Cells were recovered from the BM and SPL 16 hours after injection and were analyzed for the presence of either CFSE or CSA-SE by flow cytometry by acquiring 10^6 cells per sample using a FACSCalibur cytometer.

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PHZ treatment

Hemolytic anemia was induced with PHZ (60 mg/kg body weight; Sigma Chemical) injected intraperitoneally for 2 consecutive days.[23] The first day of injection was considered as time zero, and mice were killed at various times afterward as described. BM and SPL were removed under sterile conditions for further analyses.

Hematologic parameters

Blood was collected in heparinized microcapillaries (40 μL) from the tail vein of adult animals. Hematocrit (Hct) and reticulocyte counts were determined manually. At least 1000 cells were counted for each determination.

Reverse transcription–polymerase chain reaction (RT-PCR) analysis

Total RNA used for cDNA amplification was prepared with Trizol (Gibco, Gaithersburg, MD) following the manufacturer's protocol. For reverse transcription–polymerase chain reaction (RT-PCR) analysis, 2 μg RNA was converted into cDNA in a 20 μL reaction using the Thermoscript RT-PCR System (Gibco) primed with oligo-dT_{12-18}. Two microliter aliquots of cDNA served as template for PCR using Taq-DNA polymerase (Gibco) and specific primers for *podxl* (forward primer: GAGGATTTGTGCACTCTACATGTG; reverse primer: TACTCGAGTGGGTTGTCATGGTAACC), *cd34* (forward primer: TAGCACAGAACTTCCCAGCAAAC; reverse primer: CTCAGATCACAGTTCTGTGTCAGC), *endgl* (forward primer: ACGGGACGCTCTTTGTAGTGC; reverse primer: ATGCGGCCGCTCACAGGTGAGTGTCCTCCTC), and hypoxanthine-phosphoribosyl-transferase *(hprt)* used as a loading control (forward primer: CTCGAAGTGTTGGATACAGG; reverse primer: TGGCCTATAGGCTCATAGTG). PCR comprised 30 cycles of 94°C for 45 seconds, 58°C for 55 seconds, and 72°C for 2 minutes. PCR products were separated on a 2% agarose gel.

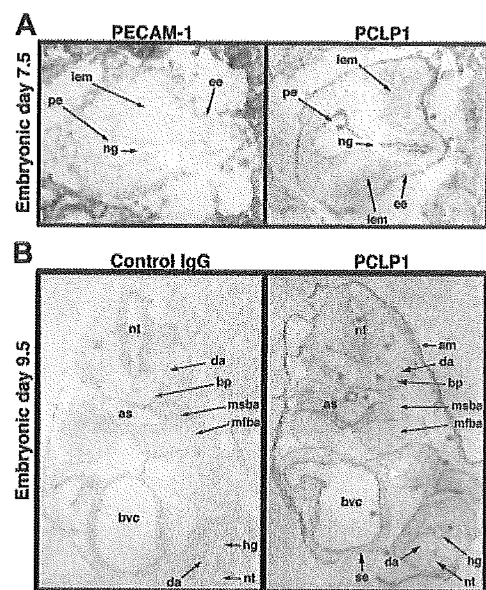

Figure 1. Podocalyxin is expressed by all 3 germ layers and boundary elements during development. Frozen sections of embryos recovered at E7.5 (A) and E9.5 (B) were stained with antibodies to podocalyxin (PCLP1) and PECAM-1 (CD31) or control antibody and counterstained with methyl green. Red arrowheads highlight cavities whose luminal/boundary faces are coated with podocalyxin. These include the primitive ectoderm (pe), embryonic endoderm (ee), amnion (am), the branchial pouches (bp), the dorsal aorta (da), aortic sac (as), bulboventricular canal (bvc), surface ectoderm (se), and neuroepithelium of the neural tube (nt). Other labeled structures include the following: ng, neural groove; iem, intraembryonic mesoderm; msba, mandibular component of second branchial arch; mfba, mandibular component of first branchial arch, hg, hindgut. Original magnification × 10 (see "Materials and methods").

Results

Podocalyxin is expressed by all 3 embryonic germ layers during gastrulation

Previous reports in avians and mice have suggested that podocalyxin is expressed by the most primitive embryonic and adult hematopoietic progenitors[12] and by hemangioblasts,[14] respectively. To characterize the spatial localization of murine podocalyxin during embryogenesis and before the appearance of hematopoietic cells, we analyzed its expression during gastrulation. Immunoperoxidase staining of transverse sections in the distal region of the embryonic pole of 7.5 dpc mouse embryos with the antipodocalyxin antibody, PCLP1, revealed expression by the first intraembryonic mesoderm (Figure 1A). Thus, the mesodermal precursors that are thought to give rise to hemangioblasts (precursors for hematopoietic cells and vascular endothelia) express podocalyxin at the primitive streak stage. More surprisingly, we found that podocalyxin was not restricted to the mesoderm but was also expressed on the apical face of the neuroepithelium in the primitive ectoderm and by the primitive endoderm surrounding the embryos. This suggests that podocalyxin may be expressed by cells of the "epiblast," which is reported to be the source of all 3 germ layers.[24] Staining of the same-stage embryos with PECAM-1/CD31 showed no reactivity. After axial rotation of the embryos and inversion of the germ layers (9.5 dpc), podocalyxin was widely expressed by "boundary elements" (vasculature, mesothelial linings, and the luminal face of newly formed cavities) as has previously been reported in avians (Figure 1B; McNagny et al[12]).

Podocalyxin is transiently expressed in all hematopoietic compartments during embryonic and perinatal life

Although the highest levels of staining were observed on mesothelial cells and boundary elements, podocalyxin was also expressed at lower levels by hematopoietic cells within the vasculature. To more accurately assess the expression levels and distribution of podocalyxin on hematopoietic subsets, we used the more sensitive technique of immunofluorescence and flow cytometry. Cell suspensions from YS, peripheral blood (PB), FTL, SPL, and BM were analyzed at various stages of embryogenesis (Figure 2). Surprisingly, while podocalyxin expression is only detectable on a very small population of cells in adult hematopoietic compartments (see "Steady state podocalyxin expression in adult BM is restricted to a subpopulation of long-term repopulating HSCs"), it is highly expressed in all hematopoietic compartments during early embryonic life, and the expression level gradually decreases during ontogeny (Figure 2A). Furthermore, the frequency of PCLP1$^+$ cells also decreases in all hematopoietic compartments during development. Essentially, all cells in day 10 YS and PB (97% ± 3%) are PCLP1$^+$, and the level of expression gradually decreases over the next 5 days of development. Similarly, podocalyxin is expressed by 75% ± 5% of day 15 FTL cells and, again, the frequency of positive cells gradually decreases over the next 3 days of development (Figure 2B). Finally, both fetal BM and SPL acquire detectable populations of PCLP1$^+$ cells (9% ± 4% and 17% ± 7%, respectively) as these tissues become hematopoietically active (E17) and, again, the frequency of positive cells gradually decreases to reach its nadir just prior to birth, with virtually all hematopoietic cells becoming PCLP1$^-$ (Figure 2B and data not shown). Strikingly, immediately after birth we observe a burst of

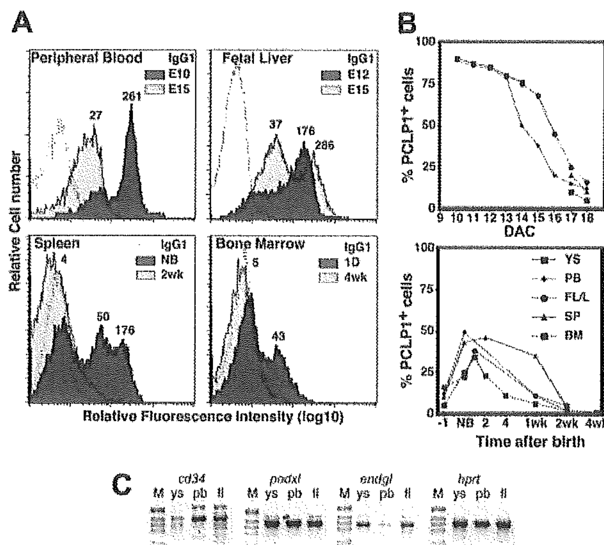

Figure 2. Podocalyxin is expressed by all primitive hematopoietic cells and then declines with development. (A) FACS profiles showing expression of PCLP1 by hematopoietic tissues at various stages of development (1 of 3 independent experiments). Mean relative fluorescence intensity (RFI) for each population is indicated numerically above each peak. (B) Graph showing the percentage of PCLP1$^+$ cells in tissues during development. DAC indicates days after coitus. (C) Expression of CD34-related transcripts in YS, PB, and FTL of E15 embryos; *podxl, endgl, cd34,* and *hprt* indicate RT-PCR–amplified products for podocalyxin, endoglycan, CD34, and hypoxanthine-guanine phosphoribosyl transferase, respectively. The RT-PCR products are shown in a negative image of an agarose gel stained with ethidium bromide. M indicates molecular weight marker.

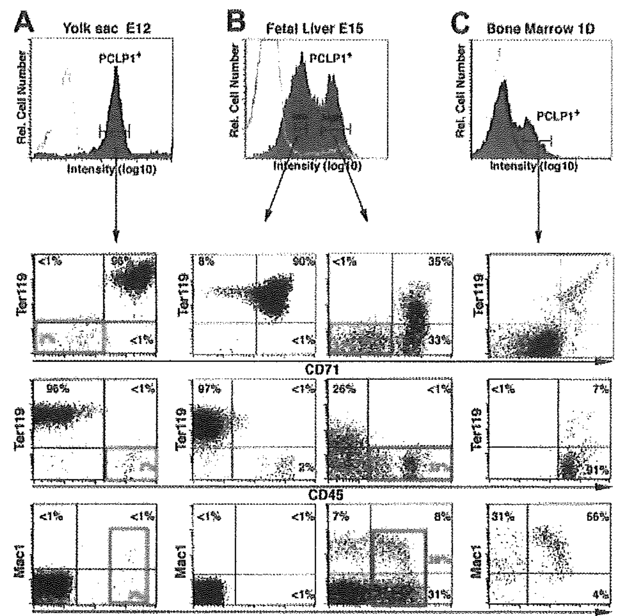

Figure 3. Podocalyxin becomes progressively more restricted to hematopoietic progenitors during embryonic development. E12 YS (A), E15 FTL (B), and 1-day BM (C) were stained with anti-PCLP1 plus antirat IgG–Alexa-fluor 488, followed by Ter119-biotin plus streptavidin-APC and CD71-PE, CD45-PE, or Mac-1–PE. PCLP1^{low} and PCLP1^{high} cells were gated as indicated, and coexpression of hematopoietic markers in each population was assessed by 3-color FACS analyses. Solid curves indicate specific staining with the indicated antibodies. Open curves indicate isotype-matched control stains. The "gated" percentages of cells in each quadrant are indicated. Red boxes indicate colony-forming cells from E12 YS. Blue boxes indicate the hematopoietic precursor fraction of 15-day FTL.

podocalyxin expression by postnatal hematopoietic tissues reaching 52% of the total cells in the SPL and more than 30% of the cells in BM (Figure 2A-B). This, again, is followed by a gradual decline over the next 2 weeks of postnatal life until podocalyxin expression is detectable only on a rare subpopulation in the SPL and BM of adult mice. No reactivity was detected in age-matched *podxl*$^{-/-}$ mice, confirming the specificity of the PCLP1 antibody for podocalyxin and not other CD34 family members (Doyonnas et al[13] and data not shown).

To independently confirm podocalyxin expression during embryonic development, we performed RT-PCR analysis to detect its mRNA (Figure 2C) and that of the related molecules, CD34 and endoglycan.[13] All 3 molecules are expressed by YS, fetal blood, and FTL (Figure 2C) and therefore could potentially compensate for each other's loss, because it has previously been shown that deletion of podocalyxin leads to up-regulation of CD34 mRNA.[13] The relative amount of podocalyxin mRNA, however, is about 10-fold and 20-fold higher than the amount of CD34 or endoglycan mRNA, respectively, in E15 YS and FTL by quantitative RT-PCR (data not shown).

In summary, our data suggest that podocalyxin expression corresponds to the onset of hematopoietic activity and the seeding of new hematopoietic compartments and that its expression gradually declines (in terms of both levels and frequency of positive cells) after hematopoietic tissue colonization.

Podocalyxin is expressed by primitive erythroid precursors and by primitive and definitive hematopoietic progenitors during embryonic and perinatal development

Most hematopoietic cells circulating between E10 and E12 are primitive, nucleated red blood cells of YS origin. The fact that nearly all the circulating cells are PCLP1$^+$ suggests that most of the nucleated red blood cells express PCLP1. This was confirmed by 2-color immunofluorescence: Most PCLP1$^+$ cells (96%) from the YS at E12 are double-positive for the erythroid markers Ter119 and CD71 (Figure 3A, lower panels). However, at this stage, there is also a small population of PCLP1$^+$ cells that is CD71$^-$ and Ter119$^-$ but positive for the stem cell factor receptor c-kit and the panhematopoietic marker CD45 (Figure 3A; red boxes). Functional analyses by colony-forming assays in vitro and CFU-S assays in vivo showed that these cells give rise to erythroid (74%) and myeloid colonies (26%) (Table 1). This suggests that, in addition to its expression by primitive erythrocytes at 12 dpc, podocalyxin is also expressed by primitive myeloid or multilineage progenitors. Moreover, no in vitro or in vivo colonies develop from PCLP1$^{-/low}$ cells in the YS, suggesting that all primitive hematopoietic progenitors express podocalyxin (Table 1 and data not shown).

While YS cells are mainly PCLP1^{high} at E12 (Figure 3A), 15-day FTL exhibits 2 distinct populations of PCLP1$^+$ cells: one PCLP1^{high} population representing about 30% of the total cells and a second PCLP1^{low} population representing more than 50% of the total cells (Figure 3B). Again, most PCLP1^{low} cells at E15 are

Table 1. Colony-forming potential of PCLP1$^+$ or PCLP1$^-$ cells from YS

	CFCs*						CFUs-S†
E12 YS	No. of wells	No. of colonies	E	GM	MK	Mix	No. of colonies
PCLP1$^-$Ter119$^-$	192	0	0	0	0	0	0
PCLP1$^+$Ter119$^-$	192	31	23	8	0	0	5

The results are expressed as a sum of 2 to 3 separate analyses.

CFCs, colony-forming cells; E, erythroid colonies; GM, granulocyte or macrophage colonies; MK, megakaryocyte colonies; and Mix, mixed colonies.

*In vitro methylcellulose CFCs from single cells seeded in 96-well plates. Number of wells analyzed and number of wells with 1 CFC is indicated.

†In vivo CFUs-S 12 days after injection of 1000 cells in irradiated recipients.

c-kit$^-$/CD45$^-$/Ter119$^+$/CD71$^+$ (90%), suggesting that they represent either previously formed primitive erythrocytes or newly formed definitive erythroblasts arising from the FTL. In contrast to the PCLP1$^+$/CD45$^-$ hemangioblasts present in the AGM,[14] 32% of the PCLP1^{high} cells found in the FTL are CD45$^+$ and therefore already committed to a hematopoietic cell fate (Figure 3B, bottom panel). In addition, while the entire PCLP1^{low} population is of erythroid origin, the PCLP1^{high} population contains all cells expressing c-kit (39% of PCLP1^{high} cells; Figure 3B, blue box), suggesting that this compartment contains all definitive hematopoietic precursors.[25] Interestingly, these PCLP1^{high} cells are negative for all mature markers (B220, CD3, and Gr-1) with the single exception of Mac-1 (Figure 3B), which is known to mark early hematopoietic progenitors/stem cells at this stage of development.[26] We conclude that in FTL at E15, the PCLP1^{high} population contains definitive embryonic hematopoietic progenitors.

To test if hematopoietic progenitors after birth continue to express podocalyxin, we examined the cell surface phenotype of PCLP1$^+$ cells in the BM (Figure 3C) and SPL (data not shown) of perinatal mice. Although most PCLP1$^+$ cells (80% to 85%) express CD71^{low}, only a low percentage (less than 10%) express the late erythroid marker Ter119 in neonatal life. Moreover, at this stage, PCLP1$^+$ cells are negative for most hematopoietic lineage markers (B220, Gr-1, and CD3), but many (60% to 70%) are c-kit$^+$ (Figure 3C). We conclude that podocalyxin is expressed by erythroblasts and early progenitors in perinatal mice.

Steady-state podocalyxin expression in adult BM is restricted to a subpopulation of long-term repopulating HSCs

To test whether podocalyxin is a marker of adult murine HSCs, we examined its expression by cells with the appropriate cell surface phenotype. BM cells from 1-week-old mice were sorted into Lin$^-$ and Lin$^+$ fractions and examined for PCLP1 expression by RT-PCR (Figure 4A). Using this method, we determined that podocalyxin is expressed exclusively by the Lin$^-$ fraction of BM known to contain HSCs. The Lin$^-$ population from adult mice (4 to 8 weeks) was then further fractionated on the basis of expression of the HSC antigens, Sca-1 and c-kit. This population, representing about 0.1% of the adult BM, is highly enriched in cells with long-term reconstituting capacity.[27] Staining of adult LSK BM cells with anti-PCLP1 revealed both PCLP1$^+$ (20%) and PCLP1$^-$ (80%) fractions (Figure 4B). Thus, in neonates and 4- to 8-week-old mice, podocalyxin is selectively expressed by a subset of BM cells with a surface phenotype consistent with HSCs.

We next tested for HSC function within LSK PCLP1$^+$ or LSK PCLP1$^-$ fractions by transplantation of these cells into lethally irradiated mice. Low numbers of these cells were isolated by fluorescence-activated cell sorting (FACS) from the BM of Ly5.1 mice and transplanted into lethally irradiated congenic Ly5.2 mice. To ensure that these mice survived the short-term, posttransplantation leukopenia following irradiation, they were coinjected with host-type (Ly5.2) radioprotective GFP$^+$ Sca-1$^-$ BM helper cells that have previously been shown to lack long-term repopulating capacity. Approximately 70% (21 of 30) of the recipients transplanted with between 10 and 500 PCLP1$^+$ LSK cells showed significant levels of hematopoietic cell chimerism (more than 0.1%) in the PB for more than 4 months after transplantation. Animals exhibiting more than 1% engraftment are shown in Table 2 (12 mice) and Figure 4D (the top 4 mice for each group). In these animals, both myeloid and lymphoid lineages developed as determined by expression of Mac-1 and B220 on donor-derived cells, thus demonstrating multilineage reconstituting capabilities of LSK

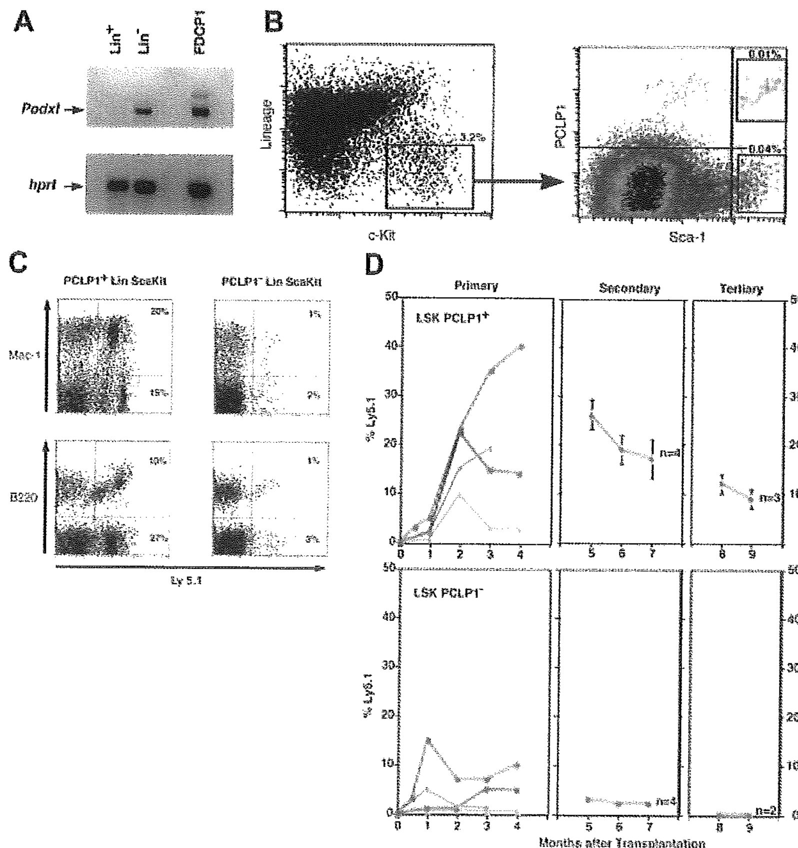

Figure 4. PCLP1$^+$ cells in adult BM are a subpopulation of HSCs. (A) RT-PCR analyses of Lin$^+$ (stem cell depleted) and Lin$^-$ (stem cell enriched) BM from 1-week-old mice and the PCLP1$^+$ cell line, FDCP-1. (B) FACS profiles showing expression of PCLP1 by a subset of the LSK HSC population in adult BM. Percentages indicate total frequency in BM. (C) Donor-derived lymphoid (B220) and myeloid (Mac-1) composition of BM 16 weeks after reconstitution of lethally irradiated mice with 500 cells of either PCLP1$^+$ or PCLP1$^-$ LSK BM cells. Ly5.1$^+$ cells are donor derived. (D) Percentage (determined by frequency of Ly5.1$^+$ staining) of donor-derived cells in the blood of mice reconstituted with PCLP1$^+$ and PCLP1$^-$ LSK cells after primary, secondary, and tertiary transplantation. In primary recipients, each colored line represents an individual mouse reconstituted with either 500, 50, or 10 enriched stem cells. In each case, 10^6 BM cells from the most highly reconstituted primary recipients (red) were used for subsequent transplantations in secondary and tertiary recipients.

Table 2. The percentage of donor-derived Ly5.1+, myeloid (Mac1+ and Gr1+), and lymphoid (B220+ and CD3+) cells in the blood of mice 8 weeks after reconstitution with PCLP1+ or PCLP1− LSK cells

No. of Ly5.1+ cells injected	No. of recipient mice	Donor-derived myeloid cells, %	Donor-derived lymphoid cells, %
PCLP1+ LSK			
500	1	3	20
50	1	1	18
10	4	2-7	0.5-20
PCLP1− LSK			
500	2	0.2-1	0.8-5
50	1	0.3	1.3
10	3	1-2	0.5-1
Controls*	3	0	0

*Control animals did not receive Ly5.1+ cells.

PCLP1+ HSCs (Figure 4C and first panel of 4D; Table 2). To further confirm the long-term reconstituting potential of these PCLP1+ cells, we analyzed their ability to contribute to the hematopoietic compartments of secondary and tertiary recipients in serial transplantation experiments (Figure 4D). A total of 10^6 BM cells from primary recipients with the highest chimerism were transplanted into lethally irradiated Ly5.2 secondary recipients. All mice analyzed after secondary and tertiary transplantation from PCLP1+ LSK HSCs had donor-derived myeloid and lymphoid PB cells (data not shown). We conclude that in BM, podocalyxin is expressed by cells with HSC activity.

Podocalyxin expression on adult erythrocyte precursors is reactivated by hemolytic anemia

Because PCLP1 expression correlates with rapid expansion of erythroid progenitors during development, we tested whether its expression could be reactivated in adult erythroid cells by hemolytic anemia. Adult mice were injected with PHZ to induce profound anemia,[28] and SPL and BM cells were monitored for PCLP1 expression at daily intervals during the recovery phase when erythropoiesis is enhanced (Table 3). During normal erythropoiesis, progenitors progress through a very well-defined set of maturational steps: from proerythroblasts (Ter119^{low}/CD71^{high}), to basophilic erythroblasts (Ter119^{high}/CD71^{high}), to polychromatophilic erythroblasts (Ter119^{high}/CD71^{med}), and finally to orthochromatophilic erythroblasts (Ter119^{high}/CD71^{low}).[29] In nonanemic mice, most Ter119^{high} cells are late orthochromatophilic erythroblasts (Ter119^{high}/CD71^{low}). We found that the PCLP1+ cells, which normally represent less than 1% of the cells in adult BM, increase rapidly after PHZ treatment and reach a peak frequency of 24% ± 4% of the total cells on the third day, well before the appearance of reticulocytes in the PB (Table 3 and Figure 5A). Moreover, we observed the expansion of PCLP1+ c-kit+ cells in BM just prior to the expansion of Ter119^{low}/CD71^{high} proerythroblasts (Figure 5B). Three-color staining revealed that PCLP1+/c-kit+ cells are predominantly Ter119− (more than 90%), while the bulk of the Ter119+/PCLP1^{low} cells are c-kit− (more than 85%, data not shown). This suggests that podocalyxin expression is rapidly increased on very early c-kit+ progenitors that can potentially emigrate from the BM to enhance extramedullary erythropoiesis. Although podocalyxin expression is largely restricted to BM, a low frequency of PCLP1+ cells is also present in the SPLs of anemic animals (Figure 5C).

Table 3. PCLP1/podocalyxin expression is reactivated by PHZ-induced hemolytic anemia, and its deficiency induces accumulation of erythrocyte precursors within the hematopoietic compartments

	Hematocrit, %	Reticulocyte index,* %	PCLP1+ cells in BM, %
PBS-treated mice			
Day 2	45 ± 5	2 ± 1	1 ± 1
PHZ-treated mice			
Day 2†	32 ± 3	4 ± 3	11 ± 2
Day 3	23 ± 4	9 ± 3	24 ± 4
Day 6	41 ± 6	67 ± 5	19 ± 3
Day 9	52 ± 4	12 ± 2	3 ± 1

The results are expressed as the mean ± SD of 3 separate analyses.

*Reticulocyte index is expressed as corrected reticulocyte count. Corrected reticulocyte count (%) = reticulocyte count (%) × 45 (normal hematocrit rate).

†Twenty-four hours after second injection of PHZ.

Podocalyxin and CD34 are required for efficient short-term homing to hematopoietic tissues

Recently, we and others have shown that podocalyxin and CD34 play an important role in blocking hematopoietic and epithelial cell adhesion and cell-cell contact.[19,20,30] Based on these observations and the fact that podocalyxin is abundantly expressed by embryonic hematopoietic cells as they seed new tissues, we postulated that these molecules may play a role in facilitating the exit and entry of hematopoietic cells into new environments. To test this hypothesis, we isolated FTL cells from *wt*, *cd34*$^{-/-}$, *podxl*$^{-/-}$, and *podxl*$^{-/-}$*cd34*$^{-/-}$ animals and assayed them for short-term homing to BM. Briefly, *wt* or mutant cells were labeled with the fluorochromes CFSE (green) or CSA-SE (red) mixed in equal proportions and injected into lethally irradiated recipient mice (Figure 6A). It has been demonstrated previously that the number of cells that home to BM in murine transplantation experiments plateaus between 3 hours and 24 hours, with no effect on apoptosis and expansion.[31,32] We therefore analyzed recipient mice 16 hours after transplantation for the relative proportion of labeled cells in the SPL and BM. To rule out the possibility of selective dye toxicity,

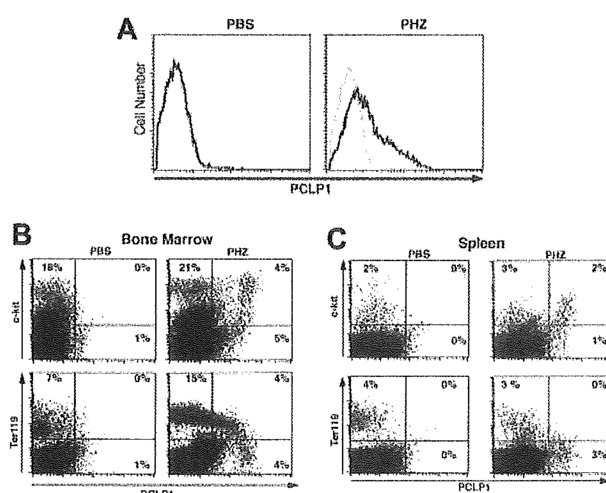

Figure 5. PHZ-induced anemia reactivates podocalyxin expression in adult erythroid cells. (A) Representative FACS histograms showing PCLP1+ cells in BM of adult mice 3 days after injection with either PBS (left) or PHZ (60 mg/kg; right). Cells were stained with PCLP1 (black line) or control mAb (gray line) and counterstained with Alexa-fluor 488–conjugated antirat IgG. (B-C) Coexpression of podocalyxin with hematopoietic progenitor and erythroid markers on BM cells from PBS- and PHZ-injected mice 2 days after the first injection. BM (B) and SPL (C) cells were stained with anti-PCLP1 as in panel A and counterstained with biotinylated Ter119 plus streptavidin-PE or c-kit–PE. Percentages are determined according to quadrant markers.

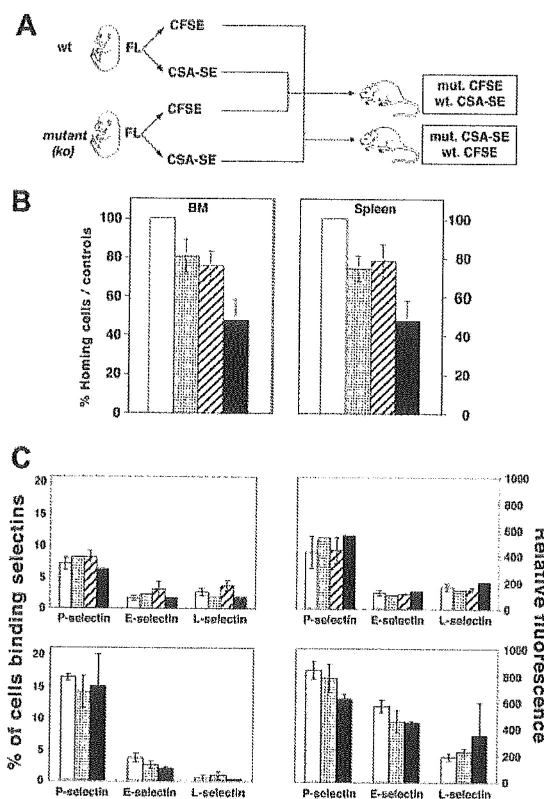

Figure 6. Impaired short-term homing in vivo despite identical selectin ligand expression. (A) Experimental design for short-term homing experiment. Mutant 15-day FTL cells were isolated, genotyped, and labeled with either CFSE or CSA-SE. These were mixed in a 1:1 ratio with *wt* cells labeled with the opposite fluorescent tracker prior to intravenous injection into irradiated recipients. Relative frequency of CFSE- and CSA-SE–labeled cells in BM and SPL was determined 16 hours later by FACS. In control experiments, the fluorescent labels were reversed to rule out dye toxicity. (B) Percent homing efficiency of *wt* or mutant FTL cells to SPL (right panel) or BM (left panel). The *wt* cells were assigned a frequency of 100%. Results from CFSE and CSA-SE homing were pooled together and standard deviation calculated for the number of mice analyzed (n = 4 per genotype). (C-D) The percentage and mean RFI of E-, P-, and L-selectin binding to *wt*, $cd34^{-/-}$, $podxl^{-/-}$, or $podxl^{-/-}cd34^{-/-}$ FTL cells. Shown are results from 2 independent experiments. (B-D) □ indicates *wt*; ▥, $cd34^{-/-}$; ▨, $podxl^{-/-}$; and ■, $podxl^{-/-}cd34^{-/-}$.

experiments were performed in duplicate and the fluorescent tags were reversed. The results of these analyses are summarized in Figure 6B. Although FTL progenitors of each genetic background (*wt*, $cd34^{-/-}$, $podxl^{-/-}$, and $cd34^{-/-}podxl^{-/-}$) were detectable in the BM and SPL of recipient mice, *wt* cells exhibited the most robust migration to both hematopoietic organs. Based on the number of cells injected and the frequency of cells recovered, we estimate that 12% of input cells were recovered in representative experiments with *wt* cells. After normalization to the number of *wt* cells harvested, the relative proportions of donor cells harvested from the BM and SPL of the recipients was determined. The $cd34^{-/-}$ or $podxl^{-/-}$ FTL cells are 20% to 30% less efficient in migrating to the SPL and BM of recipient mice (Figure 6B). Loss of both CD34 and podocalyxin had an additive effect, and these cells are less efficient in BM and SPL homing (Figure 6B).

Previously, it has been shown that, when expressed by high endothelial venules, podocalyxin and CD34 are glycosylation-dependent adhesive ligands for L-selectin on migrating leukocytes. Although the ability of L-selectin to bind to CD34 and podocalyxin is dependent on an unusual lymph node–specific glycosylation that has not been observed on most vasculature or hematopoietic cells, it remained a formal possibility that the defects observed in progenitor cell homing could be due to loss of these molecules as selectin ligands. To rule out this possibility, FTL from *wt*, $cd34^{-/-}$, $podxl^{-/-}$, and $podxl^{-/-}cd34^{-/-}$ embryos were stained with recombinant, soluble forms of P-, E-, and L-selectin, the only molecules known to play a role in carbohydrate-dependent adhesion of hematopoietic cells. The results are shown in Figure 6C-D. Whether stained individually or with a cocktail of soluble selectins, we found no significant difference in the frequency or the level of selectin ligand expression by *wt* or mutant FTL cells. We conclude that the short-term homing defects observed with $cd34^{-/-}$, $podxl^{-/-}$, and $podxl^{-/-}cd34^{-/-}$ cells are not due to the loss of these molecules as selectin ligands and are more likely a result of these molecules providing a "Teflon" effect in preventing nonspecific adhesion.

Interestingly, although hematopoietic progenitors from double-deficient mice seem to be highly impaired in their ability to migrate and home to hematopoietic tissues, they retain their competence for normal differentiation into mature hematopoietic lineage cells. Indeed, we have found that injection of between 2.5×10^5 and 10^6 double-deficient FTL cells is sufficient to fully reconstitute lethally irradiated recipient mice. Likewise, we have seen no detectable differences in the frequencies or subsets of hematopoietic lineage cells in the FTL of single- or double-deficient embryos (Doyonnas et al[13] and data not shown). Thus, despite their impaired short-term migration, the data suggest that, when provided in sufficient numbers and given sufficient time, these cells are able to find the appropriate niches for proliferation and maturation. In summary, our preliminary data suggest that CD34 and podocalyxin have overlapping roles in aiding the short-term migration of hematopoietic progenitors.

Discussion

During ontogeny, sequential changes in the anatomic sites of hematopoiesis are believed to occur through the migration of hematopoietic stem and progenitor cells from the extraembryonic YS and/or the AGM into the FTL, the SPL, and the BM of developing embryos. Evidence suggests that most migrating embryonic hematopoietic progenitors are contained within the cell fraction expressing the sialomucin, CD34.[33,34] Despite the use of this molecule as a selective marker for hematopoietic precursor cells, deletion of the encoding gene in mice does not appear to significantly perturb the migration of hematopoietic progenitors during embryogenesis or in adult life.[35,36] We and others have reported the discovery of a new family of CD34-related molecules,[12,13,16,18] and gene-targeting studies have suggested that members of this class of sialomucins may functionally compensate for each other's loss in tissues where they are coexpressed.[13] In the present study, we have analyzed the hematopoietic expression of one of these new family members, podocalyxin, during ontogeny. The results suggest that podocalyxin, in contrast to CD34, is expressed by all 3 embryonic germ layers during gastrulation and that its expression is maintained on both primitive and definitive embryonic hematopoietic progenitors. Moreover, we find that podocalyxin is also expressed by nucleated red blood cells and their precursors.

At the earliest stages of development (E8 to E12), podocalyxin is expressed by essentially all circulating hematopoietic cells (97% ± 3%), including all cells capable of forming colonies in vitro and in CFU-S assays. Previous reports have suggested that these cells express the progenitor cell antigens CD34, Sca-1, and c-kit; consistent with this, we find that all cells expressing these markers coexpress podocalyxin. Similarly, we find that early

hematopoietic progenitors in the FTL, SPL, and BM also express podocalyxin. By 2 weeks after birth, podocalyxin is nearly undetectable in BM, while a sizable population retains expression of CD34 and c-kit (more than 5%) and, as published previously, these cells contain the majority of colony-forming units. Here we show that the few remaining $PCLP1^+$ cells (less than 0.5%) in adult BM contain long-term repopulating HSCs with the capacity to reconstitute lethally irradiated primary, secondary, and tertiary recipients. All of the experiments described here were performed with mice not older than 8 weeks. Notably, CD34 has been shown to be a marker of long-term repopulating cells only until 10 weeks of age.[37] Subsequently, most long-term repopulating cells are $CD34^-$. CD34, however, is reexpressed by these cells after mobilization with G-CSF or after transplantation (reviewed by Ogawa[38] and Zanjani et al[39]). Thus, the data suggest that CD34 expression probably reflects the activation/cell cycle status of long-term repopulating cells. Interestingly, we have found that podocalyxin continues to be expressed by the LSK population in the BM of older mice (more than 10 weeks), although we have not yet shown that these cells have long-term repopulating activity. Thus, the mechanisms that regulate the expression of podocalyxin and CD34 by stem cells may differ.

In addition to its expression by hematopoietic progenitors, we have shown by cytologic, flow cytometric, and functional analyses that most cells expressing podocalyxin during embryonic and neonatal life are affiliated with the erythroid lineage. Thus, podocalyxin marks essentially all nucleated erythroid cells at the earliest stages of hematopoietic development and the bulk of the definitive erythroid progenitors in FTL and neonatal BM and SPL. Our data suggest that expression of podocalyxin by these cells (as well as hematopoietic progenitors) correlates closely with high rates of erythropoiesis and with the expansion and seeding of erythroid progenitors to new hematopoietic microenvironments. Thus, 10-day YS, 15-day FTL, and newborn BM and SPL each exhibit a burst of podocalyxin expression by erythroid progenitors that declines as erythropoiesis shifts to a new anatomic site or reaches steady state. Moreover, we have shown that podocalyxin expression is rapidly reactivated by erythropoietic stress.

Interestingly, erythropoiesis during development is in many ways analogous to stress-induced erythropoiesis, because embryos sustain a much higher rate of red cell production to compensate for their lack of a reservoir of erythropoietic progenitors.[40,41] It therefore appears that podocalyxin is expressed by early erythroblasts only in situations where maximal red cell output is essential. Several genes have been described that appear to be critical for fetal erythropoiesis but not for steady-state erythropoiesis in adults. These include the transcription factors activating transcription factor 4 (ATF4), adenoviral E2 gene promoter-binding factor 4 (E2F4), and signal transducer and activator of transcription 5a/5b (Stat5a/5b).[42-44] Inactivation of each of these genes results in severe anemia during neonatal and fetal life but relatively normal erythropoiesis in adults. Interestingly, most of these deficiencies lead to increased sensitivity to chemically induced anemia, further suggesting a deficiency in response to erythropoietic stress. It will now be interesting to see if the expression of any of these factors correlates with the high level of podocalyxin expression on embryonic erythroid cells or the lower level observed on PHZ-treated erythroid cells. Alternatively these differences could correlate with turnover rate of this antigen on embryonic versus adult erythroid cells. The induction of podocalyxin expression does not appear to be hypoxia inducible, because preliminary data showed treatment of $PCLP1^+$ cell lines (Baf3 or FDCP-1) or primary BM cells with desferroxamine or CoCl had no effect on podocalyxin expression (although they did augment CD71 expression). Likewise, we were unable to detect a change in expression in mice treated with 5-fluorouracil (5-FU). Thus, the mechanism of podocalyxin up-regulation during anemia will require a more detailed analysis of the gene's regulatory elements.

How do CD34-related proteins aid in hematopoietic cell migration? Recently it has been shown that ectopic expression of podocalyxin in adherent cells leads to a profound block in cell-cell adhesion.[20] For example, overexpression in CHO and MDCK cells (in vitro) was found to result in an expression level–dependent block in homotypic aggregation and to inhibit the formation of cell-cell tight junctions, respectively.[20] Likewise, we have found that podocalyxin is selectively up-regulated in metastatic breast cancers with poor outcome and that ectopic expression in more benign breast cell lines leads to loss of polarity and cell-cell junctions.[19] Conversely, we have shown that deletion of the podocalyxin-encoding gene in mice leads to perinatal lethality due to excessive cell-cell adhesion in a number of embryonic structures. For example, podocalyxin loss from the developing mesothelium leads to excessive adhesion between the embryonic intestine and the umbilical cord, resulting in herniation of the gut in many embryos. In addition, all mice lacking podocalyxin die perinatally due to high blood pressure and kidney failure from excessive adhesion and failure to remodel tight junctions between kidney podocytes.[13] Finally, we have recently shown that mast cells selectively express CD34, and deletion of the encoding gene leads to homotypic mast cell aggregation.[30] Thus, all of the available functional data suggest that podocalyxin and CD34 play a direct role in blocking cell-cell adhesion.

Based on these observations and the abundant expression of podocalyxin by boundary elements early in development (Figure 1) we propose that, on hematopoietic progenitors, podocalyxin and CD34 perform a similar function and act as molecular Teflon to enhance the ability of these cells to cross endothelial barriers and enter/exit primary hematopoietic tissues. This is supported by short-term homing assays that reveal a gene dose–dependent impairment in the ability of cells to enter the BM. That these molecules function as invasive factors rather than specific homing receptors is supported by the observation that *wt* and mutant cells show an identical ability to bind selectins, which are the only known receptors for these molecules.

Although CD34 and podocalyxin are both expressed by hematopoietic progenitors, only podocalyxin is expressed by most erythroid cells during embryonic development and in response to anemia. We postulate that the high-level expression in YS and subsequently FTL may reflect a role in the migration of erythroid progenitors and more mature erythroblasts through the endothelial barriers in these tissues and into new sites of erythropoietic activity. In the YS most of the inner cells of the blood island progressively lose their intercellular attachments as they differentiate into primitive erythroblasts and then leave these anatomic sites. Likewise, in the FTL, hematopoietic precursors cross the endothelial barrier of the vasculature to reach the PB, where they can circulate freely and later seed the developing BM and SPL. Finally, severe anemia leads to an efflux of erythroid progenitor cells from the BM and to the establishment of extramedullary sites of erythropoiesis.[44,46] Although these phenomena have been well documented, the molecular mechanisms governing the release of cells from these sites has not been clarified. It will now be interesting to see if podocalyxin, by acting as molecular Teflon, plays an important role in this process, and this work is in progress.

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基礎研究

哺乳類の胚発生における造血と成体型造血幹細胞の起源

Origin of hematopoiesis and definitive hematopoietic stem cells in mammalian embryogenesis

Key point

◉哺乳類では最初に胚体外の卵黄嚢で胎児型造血が起こり，続いて胚体内のAGM領域で成体型造血幹細胞が発生する．さらに，造血機能は発生の進行とともに肝，脾，骨髄へと移行する．

◉哺乳類の造血系の発生は両生類や鳥類と類似性が高い．

◉哺乳類においては胎盤も造血組織として機能することがあらたに報告され，注目を集めている．

◉胎生期の造血は，造血器官の移行や，それに伴う造血細胞の変化など，成体の造血と異なる点があり，今後の解明が期待される．

成体型造血幹細胞はいつ，どこで，どのように生じるか

成体の血液に含まれる赤血球，血小板，顆粒球とリンパ球を含むさまざまな白血球は，骨髄に少数存在する造血幹細胞(hematopoietic stem cell：HSC)から分化し，比較的短い寿命(ヒトの場合，赤血球で約120日，好中球で約4日)で入れ代わる．このため骨髄では大量の血球を供給し続ける必要があるが，造血の起源となる造血幹細胞は枯渇しないことから，造血幹細胞は自己複製能とさまざまな血球へ分化できる多分化能をもつと考えられる．遺伝的背景が均一な純系マウスを用いると容易に移植実験が行えるため，造血幹細胞の研究は哺乳類ではマウスをモデル動物として進展してきた．現在もっとも厳密な成体型造血幹細胞の定義は，致死量放射線照射により造血系を破壊した成体マウスに移植すると4カ月以上の長期にわたり血球の全系譜へ分化できる細胞(long-term repopulating HSC：LTR-HSC)とされている．このような血球および造血幹細胞は個体発生において，いつ，どこで，どのように出現するのであろうか．この問いに対する答えを求めて，これまで多くの研究者がさまざまなモデル動物を用いて解析を行ってきた．

鬼塚和泉，竹内眞樹，宮島　篤／東京大学分子細胞生物学研究所機能形成研究分野
Izumi ONITSUKA, Masaki TAKEUCHI and Atsushi MIYAJIMA

哺乳類における造血幹細胞の起源

脊椎動物の造血系は中胚葉組織に由来する．マウスでは胎生6.5日(E6.5)からE7.5にかけて外胚葉層と内胚葉層の間に中胚葉層が誘導される．この中胚葉から血球および血管はこれらに共通の前駆細胞であるヘマンジオブラストを経由して分化すると考えられている[1)]．最初の造血はE7.5に胚体外の卵黄嚢の血島において，おもに有核で胎児型ヘモグロビンをもつ胎児型赤血球とマクロファージとを産生する胎児型造血(Primitive Hematopoiesis)としてはじまる．一方，赤血球，血小板，白血球の3系統を産生する成体型造血は主として胎児肝が担うが，両生類や鳥類の胚を用いた移植実験により成体型造血幹細胞の起源は肝ではなく腹部大動脈周辺の組織に由来することが示されていた．哺乳類においては1990年代半ばまで造血系の起源は卵黄嚢であり，造血機能は卵黄嚢から肝へと直接移行するものと考えられていた．しかし，両生類，鳥類と同様に哺乳類でも肝で造血がはじまる以前の腹側大動脈を含む領域に，成体型造血幹細胞の起源があることをはじめて示したのがGodinらとDzierzakらである．GodinらはE8.5〜9マウス胎仔の腹部大動脈周辺の傍大動脈臓側中胚葉(paraaortic splanchnopleural mesoderm：PAS)と名づけた領域をSCIDマウスの腎周囲に移植し，数カ月後にホストの血液中にドナー由来のIgM, IgMを産生するB細胞および形質細胞を検出した[2)]．したがって，胎仔肝での造血がはじまる12時間前のPAS領域に，成体型造血の一部であるリンパ球系の血球を産生する前駆細胞が存在することが示された．

一方，DzierzakらはPAS領域とほぼ同じ領域で発生段階的にわずかに遅い時期の大動脈-生殖隆起-中腎(aorta-gonad-mesonephros：AGM)領域について造血前駆細胞活性(「サイドメモ」参照)の有無を検討した[3)]．彼らは肝で造血がはじまる以前のE9のAGM領域にすでに造血前駆細胞が局在していることを見出し，これが血流を介して肝へ移行し生着していると考えた．彼らはさらに，卵黄嚢とAGM領域に存在する造血前駆細胞のLTR-HSC活性を検討した[4)]．E8，E9では卵黄嚢，AGM領域の細胞のいずれを移植してもドナー由来の細胞は生着しなかった．E10になると，卵黄嚢の細胞はやはり生着しないのに対しAGM領域の細胞はホストマウス100匹中3匹という低頻度ながらドナー由来の細胞が全系譜に生着し，移植後8カ月経過してもホストの血液系はすべての造血組織・造血系統で高率にドナー由来細胞に置き換わっていた．

E11 の AGM 領域には E10 より多数の造血幹細胞が含まれており，LTR-HSC 活性を有する造血幹細胞が高率に存在することが示された．この時期の胎仔から卵黄嚢や胎仔肝を移植した場合も LTR-HSC 活性がみられたが，その寄与率は AGM 領域より低かった．以上の結果からマウスにおいても両生類や鳥類と同様に，成体型造血幹細胞は AGM 領域に最初に出現することが明らかとなった．

それでは胚体外と胚体内の 2 カ所にみられる造血前駆細胞はたがいにどのような関係にあるのであろうか．卵黄嚢-胎仔間の循環系は，E8.5 ですでに確立していることから，これら 2 カ所の造血前駆細胞は，どちらか一方の部位で発生して循環系を介して他方に到達したか，マウス胎仔内の別の部位で発生してこれらの部位へ到達した，あるいはそれぞれの部位で独立に発生する可能性が想定される．これに対して，Dzierzak らはあらかじめ数日間の器官培養を行うことで，組織間の細胞の移行を除外しそれぞれの組織に由来する造血前駆細胞の活性を検討できると考え，卵黄嚢，AGM 領域，肝を器官培養した後に移植を行った[5]．その結果，LTR-HSC 活性をもつ造血幹細胞は，E9 の卵黄嚢，AGM 領域，肝にはまったく検出されず，E10 では AGM 領域にのみ検出された．E11 では卵黄嚢，AGM 領域，肝のいずれにも LTR-HSC 活性がみられた．

これらの研究から，マウスでは AGM 領域が成体型造血幹細胞の発生部位と考えられるようになった．この発見により他の脊椎動物と同様に哺乳類においても胎児内部で造血幹細胞が出現することが示され，両生類，鳥類および哺乳類で造血系の発生過程に高い類似性があることが明らかとなった．

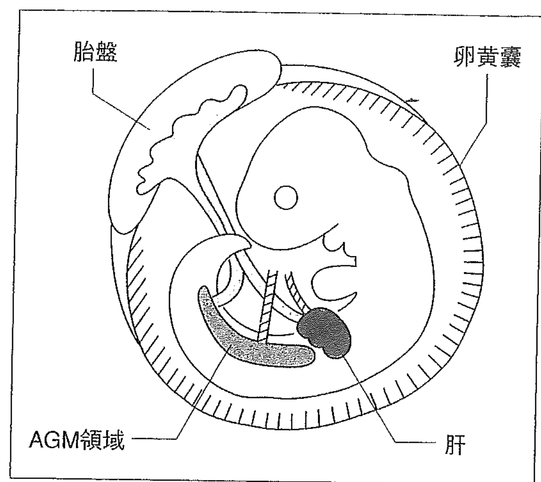

図 1　E11.5マウス胎仔の造血部位

この時期では卵黄嚢，AGM 領域，肝，胎盤の 4 カ所で成体型造血幹細胞が検出される．AGM 領域および肝は，それぞれ臍帯動脈と臍帯静脈で胎盤と，また卵黄動脈と卵黄静脈で卵黄嚢とつながっている．肝では赤血球を中心とした造血と成体型造血幹細胞の増幅が起こる．

> **サイドメモ**
>
> **造血前駆細胞活性の検討法**
>
> 試料中の造血前駆細胞活性を検討する一般的な手法として血球コロニー形成法(colony forming unit-culture：CFU-C)と脾コロニー形成法(colony forming unit-spleen：CFU-S)がある．前者は各種サイトカインを含む半固形培地中で試料細胞を培養し，形成された血球コロニーの数と形態から，もとの試料に含まれていた造血前駆細胞の数や性質を推定する方法である．後者は致死量放射線照射したマウスに同系マウスの造血前駆細胞を尾静脈より移植し，8～14 日目に脾に形成されるコロニーの数を測定する方法である．どちらも造血幹細胞の厳密な検出はできない．最近では専用の半固形培地が市販されて，より簡便なコロニー形成法が主流となってきている．

造血幹細胞活性に対する造血環境の重要性

Dzierzak らの精力的な研究によって成体型造血幹細胞の起源は AGM 領域にあると決着したかにみえた．ところが，1990 年代後半になって卵黄嚢の細胞でも新生仔の肝に移植した場合には長期にわたり多分化能と自己複製能をもつ造血幹細胞活性が認められることを Yoder らが報告した[6]．彼らは，妊娠マウスの腹腔内に抗癌剤の busulfan を投与して胎仔の造血幹細胞を減少させ，造血機能の残存する出生直後の肝に E9 の卵黄嚢細胞を直接移植した．すると，同じ細胞を成体に移植する場合とは異なり，長期間の造血幹細胞活性を示したのである．この結果は卵黄嚢の細胞も新生仔肝の造血環境を通過することで成体型造血幹細胞活性を獲得しうることを示しており，卵黄嚢細胞は成体型造血に寄与しないというそれまでの定説を覆すものであった．この報告をきっかけに，成体型造血幹細胞の起源はどこかという問題はふたたび議論の対象となった．

あらたな造血組織の発見

さらに近年，哺乳類においては，卵黄嚢，AGM 領域，肝に加え，胎盤も胎生期の主要な造血組織として機能することが報告された[7-9](図 1)．2003 年に Alvarez-Silva らは鳥類の尿膜(Allantois)が造血活性を有することから，尿膜と胎盤外膜円錐(ectoplacental

cone)が融合して漿尿膜胎盤(chorioallantoic placenta)を形成する哺乳類では胎盤でも造血が起こるのではないかと考えた．彼らはE8.5のマウス胎盤に胎仔性の造血前駆細胞を検出し，以後胎生後期にかけて胎盤細胞が卵黄嚢や肝と同等以上に高い造血前駆細胞活性を保持していることを示した[7]．2005年，Gekasらは E9.5からE18.5の胎盤細胞を成体へ移植し，その造血幹細胞活性を卵黄嚢，AGM領域，胎仔血，肝と詳細に比較した[8]．その結果，成体型造血幹細胞はAGM領域とほぼ同時期のE10.5の胎盤においても検出されはじめ，その数は肝での増幅と同調してE11.5～13.5の間に急上昇し，以後肝の造血幹細胞数が最大になるE15.5にかけて急速に低下した．これらの胎盤由来造血幹細胞は二次移植においても造血再建活性を保持していたことから，高い自己複製能をもつと考えられる．さらに，興味深いことに，肝や胎仔血中にまだ造血幹細胞が検出されないE11.0において胎盤ではすでに造血幹細胞活性が明確に認められた．これらの結果は，胎仔血中の造血幹細胞を胎盤で検出したのではなく，造血幹細胞が胎盤で発生し，胎盤も肝と同様に造血幹細胞の増殖を支持可能な造血環境を備えている可能性を示唆している．

一方，Ottersbachらは造血幹細胞マーカーのひとつであるSca-1遺伝子のプロモーターの下流にGFPを結合したトランスジェニックマウスを用いて胎盤に成体型造血幹細胞が存在することを見出した[9]．彼らも移植実験によりGekasらと一致した結果を示した．造血幹細胞はE9およびE10の胎盤からは検出されないが，E11になると多分化能を有し二次移植も可能な成体型造血幹細胞が検出され，すくなくともE12以降まで増幅した．また，胎盤の造血幹細胞はすべてGFPを発現していたことから，これらはすべてSca-1陽性細胞であると考えられた．彼らはさらに，免疫組織染色法により胎盤内の造血幹細胞の存在部位を詳細に解析し，造血幹細胞は漿膜およびラビリンス領域など，胎仔側の管腔構造の内皮細胞の存在部位に一致して存在することを見出した．

造血幹細胞がまったく独自に胎盤で発生するのか，あるいは血流を介して他の組織から移行したのかについてはいまだ決定的証拠は得られていない．胎盤は胎生期において母体-胎児間の酸素および栄養分の交換を担い，哺乳類で特異的に発達した臓器であることから，卵生の動物とは異なる造血様式を哺乳類が獲得した可能性も考えられる．これらの未解決の問題については今後の解明が期待される．

おわりに

このように，脊椎動物の発生初期における造血機構は，近年その理解が著しく進んだ結果，成体のそれとは相違点があることも明らかになってきた．これまで詳細に解析された成体骨髄の造血は，造血系が完成した個体のホメオスタシス維持のために，緩やかに分裂する骨髄造血幹細胞から安定的な血球産生が長期間維持される．一方，胎生期では胎児の発達とともに血球系と心血管系からなる循環系自体が急速に形成される．胎生期の中心的な造血器官である胎児肝では造血幹細胞活性が急激に増加しつつ赤血球系に強く偏った造血が一過性に行われ，同時に肝自体の臓器形成が進行する．この間に，胸腺・脾・骨髄という成体でも造血器官として機能する臓器も並行して発達する．こうした成体と胎生期の造血系のさまざまな相違点の解明は今後に残された課題であり，その解析を進めることでわれわれの身体の隅々までいきわたる巨大な臓器である循環系の全貌がより明らかになると考えられる．

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肝臓における造血ニッチ
—胎生期造血—

竹内眞樹* 宮島 篤*

造血系は，造血ニッチとよばれる支持細胞環境のなかで，造血幹細胞から多彩な血球を産生する複雑な細胞システムである．成体では骨髄で造血がおこなわれるが，胎児期は肝臓が中心的な造血器官となる．多種類の血球を長期安定的に供給する骨髄造血とは大きく異なり，胎児肝臓の造血は一過性で，急激な造血幹細胞の増殖を伴って赤血球系にかたよった血球産生がおこなわれる．この間に，造血ニッチを含む胎児肝臓自体も造血器官から代謝臓器へと変化を遂げる．本稿ではマウスでの知見を中心に，まず造血系および肝臓の発生の初期段階を要約し，つぎに成体骨髄とは異なる点が多い胎児肝臓における造血の特徴について述べ，さらに胎児肝臓の造血ニッチについて論じる．

はじめに

造血系は，造血幹細胞（hematopoietic stem cell：HSC）からさまざまな機能をもつ成熟血球を産生するために構成されたきわめて複雑な細胞システムである．成体哺乳類では，骨髄が生涯に渡る造血の場となるため，造血機構の研究は骨髄を中心におこなわれてきた．骨髄では少数のHSCが緩やかに分裂し自己複製と造血前駆細胞への分化をおこない，旺盛な増殖能をもつ造血前駆細胞から多くの中間段階を経てさまざまな成熟血球が安定的に産生され，血液中へ放出される．HSCの自己複製と分化は，骨髄の造血ニッチ（niche）とよばれる特別な造血環境で，骨芽細胞などの造血支持細胞や細胞外基質との接着およびサイトカインなどの液性因子によって制御されていると考えられている[1)~3)]．一方，発生過程では，胎児肝臓が中心的な造血の場であり造血ニッチを形成している．多種類の血球を長期間安定的に供給する骨髄造血とは大きく異なり，胎児肝臓の造血は一過性で，急激なHSCの増殖を伴って赤血球系にかたよった血球産生がおこなわれる．この間に，造血ニッチを含む胎児肝臓自体も造血器官から代謝臓器へと変化を遂げる．

Key Words
成体型造血
造血幹細胞
骨髄
造血ニッチ
肝芽細胞

血液学はマウスをモデル動物として研究が進められてきた．マウスでは抗体を用いたフローサイトメトリー（flow cytometry：FCM）法による細胞表面抗原の解析やサイトカインなどによる細胞の増殖・分化能の*in vitro*および*in vivo*のさまざまな解析が可能である．

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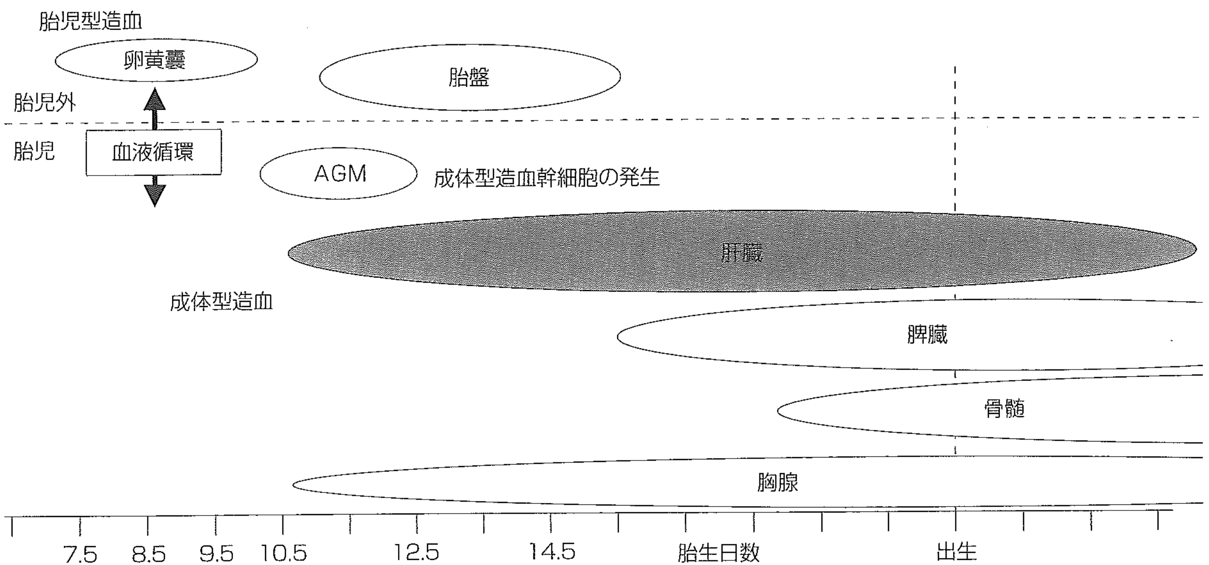


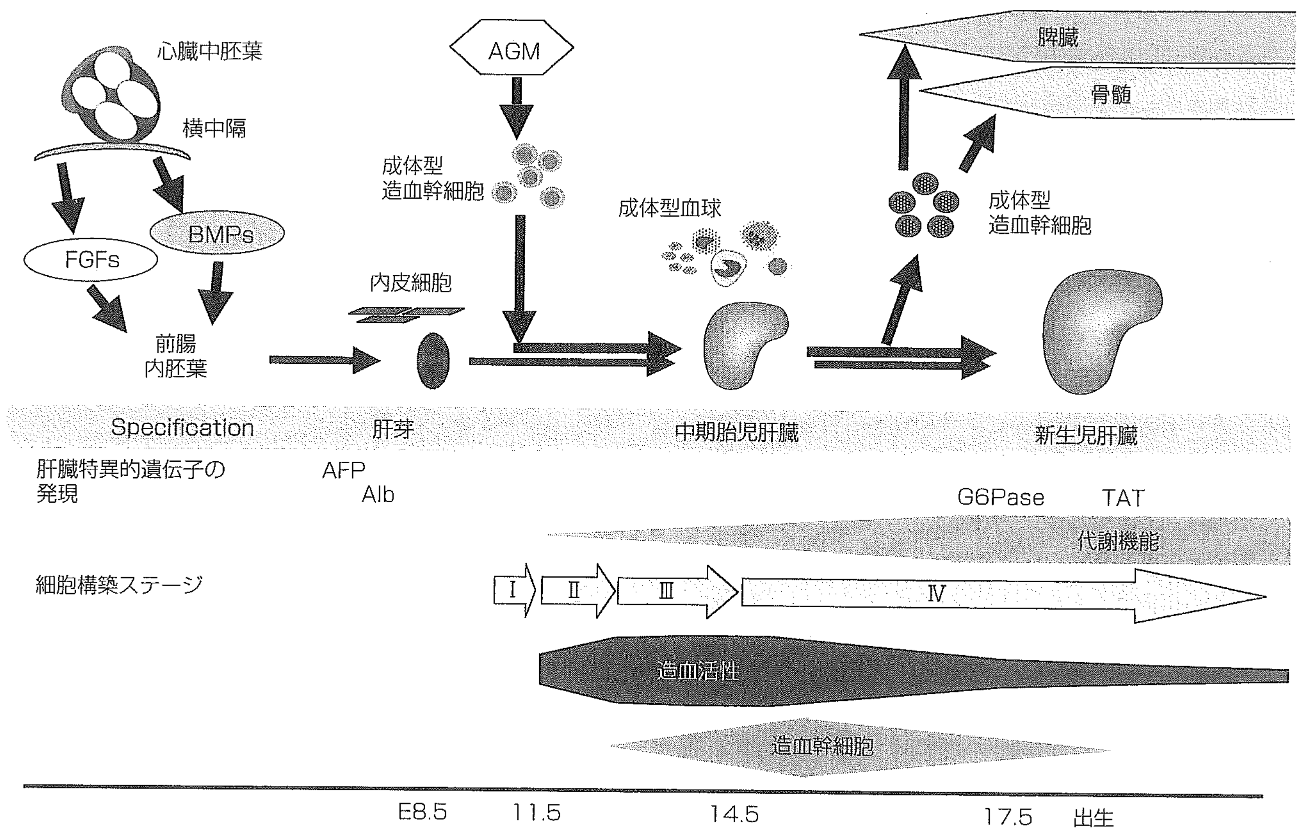
図❶　マウスにおける造血部位の移行（筆者作成）
マウスの発生過程において最初の造血は，胎児外の卵黄嚢で有核の赤血球を産生する胎児型造血として認められる．その後，胎児内部の腹部大動脈を含むAGM領域で成体型造血幹細胞が発生し，速やかに肝臓に移行する．成体ではさまざまな代謝の中心臓器である肝臓は，胎児期では中心的な造血器官として機能し，核をもたない成体型赤血球を中心とした盛んな造血と造血幹細胞の著しい増殖がおこなわれる．肝臓が次第に代謝臓器としての機能を獲得するのに伴って，造血器官としての機能は低下し，出生前後では骨髄での造血がはじまり，生涯にわたって血球が供給される．最近になって胎盤も成体型造血器官として機能することが報告されており，AGMおよび胎児肝臓との関連が注目されている．

とくに純系マウス間では拒絶反応なく移植できることから，致死量の放射線を照射して内在性のHSCを不活化した後，外来性の造血前駆細胞を移植して，すべての系譜の血球への分化能が数ヵ月間以上持続することを検討することでHSCを検出し厳密に定義できる（long term repopulating-HSC：LTR-HSC）．さらにマウスは，任意の段階の胎児組織が得られ，遺伝子改変マウスを利用できることからも血液学研究に強力なモデルとなる．

本稿ではマウスでの知見を中心に，まず造血系および肝臓の発生の初期段階を要約し，つぎに成体骨髄とは異なる点が多い胎児肝臓における造血の特徴について述べ，さらに胎児肝臓の造血ニッチについて論じる．

1．造血系の発生

造血系は，胎児期にいくつかの部位を移行しながら発達する（**図❶**）．最初の造血は，マウスでは胎生7.5日（E7.5）ごろに胎児外の卵黄嚢で血管内に有核の胎児型赤血球を産生する血島（Blood Island）で胎児型造血が観察される．E8.0まではさらに多くの胎児型赤血球が産生され，E9以降には胎児本体を循環する．E8からE10の卵黄嚢細胞がリンパ球系および赤血球系さらには多分化能をもつ造血前駆細胞へ寄与し[4)5)]，またE9卵黄嚢細胞を薬剤処理した新生児肝臓へ移植して成体型造血をおこなうことが報告されているが[6)]，成体への移植でLTR-HSC活性はE11以前の卵黄嚢では検出されない[7)8)]．一方，胎児本体ではE10.5の大動脈-生殖原基-中腎（aorta-gonad-mesonephros：AGM）領域ではじめてLTR-HSCが検出される．このように胎児外の卵黄嚢と胎児内のAGM領域の2ヵ所に造血細胞の起源部位があるが，どちらの組織でも造血細胞の初期発生は血管と密接に関連していることから，血管内皮細胞と血液細胞が共通の前駆細胞であるヘマンジオブラストから分化すると考えられている．造血細胞，とりわけ成体型HSCの発生機構に関しては，現在もさまざまな議論がつづいている．AGM領域でHSCが検出された直後から胎児肝臓でもHSCが検出されて，脱核した成体型赤血球を中心としたさまざまな種類の血球を大量に産生する成体形造血がおこなわれるとともに，HSCが著しく増幅される．



図❷ マウスにおける肝臓の発生と造血(筆者作成)

その後，脾臓，骨髄でも造血がおこなわれ，出生後には骨髄が造血の中心となる．

2．肝臓の初期発生

成体の肝臓は体内で最も大きな内臓であり，栄養物の加工，代謝産物や血漿蛋白質の維持，解毒などのさまざまな機能をもつ．これらの機能は，肝臓の約80％を占める肝細胞(hepatocyte)が担っている．肝臓の機能は特異な肝循環のうえに成り立っており，そのため肝臓はきわめて特殊な血管構築をもつ．腸管で吸収した栄養素を含んだ静脈血が門脈を通って肝臓内へ入り，肝動脈からの酸素を含む血液と混合されて類洞とよばれる毛細血管を通り中心静脈から流出する．肝細胞は1層に並んで肝細胞索を形成し，類洞がこれを挟んでいる．このように成体の肝臓は，類洞という毛細血管の巨大な塊といえる．一方，胎児期の肝臓はまず造血器官としての構造と機能をもち，次第に代謝器官としての構造と機能を形成する．

肝臓の発生過程は関与する分子および機能によっていくつかの段階に分けることができる(**図❷**)[9]．マウスでは，E6.5前後で原腸陥入が起こり，外胚葉，中胚葉，内胚葉の三つの胚葉が形成される．肝臓形成の最初の段階はE8.0(6体節期)前後に起こる前腸内胚葉の分化決定(Specification)であり，肝臓特異的な蛋白質であるアルブミンおよびαフェトプロテインの発現で特徴づけられる．Zaretらの器官培養を用いた詳細な研究[9]により，心臓中胚葉由来のFGFと横中隔由来のBMPが肝臓への分化決定を誘導することが示された．肝細胞へ分化決定した細胞は，E8.5からE9.0の間に内胚葉性上皮のなかで増殖する．E9.5になると，これらの肝細胞は横中隔の中胚葉のなかに移行して肝原基を形成する．肝原基には肝細胞と胆管上皮細胞へ分化する肝芽細胞が存在する．E10.5になると血液細胞の肝臓への進入が明瞭になり，これ以降，胎児肝臓は胎生後期の中心的な造血臓器として機能する．周産期になると，肝細胞はグルコース6-ホスファターゼ(G6Pase)やチロシンアミノトランス

フェラーゼ(TAT)およびチトクロームP450系などの成熟肝臓の機能に関与する遺伝子を発現する一方で造血支持機能が低下し，構造的にも肝細胞索と類洞からなる特徴的な細胞構築を形成するようになる.

3. 胎生期肝臓における造血の特徴

成体型HSCはAGM領域で発生するが，成体型造血としての多系統の血球産生は肝臓でおこなわれる[10]. このことは成体型造血には造血ニッチを必要とすることを示唆している. マウスでは，肝臓での造血はE10.0で検出できるようになり，出生後第1週まで持続する(図❶). この間に，胎児肝臓に含まれる血液細胞は質的にも量的にも変化をつづける一方，肝臓自体も主要な造血器官から代謝の中心臓器へと大きく機能を変化させていく(図❷).

成体骨髄での造血と比べて胎児肝臓造血は著しく赤血球産生にかたよっている. マウス赤芽球の表面抗原であるTER-119陽性細胞の比率は，成体骨髄では20～25%なのに対してE14胎児肝臓では80～90%と大部分を占め，新生児肝臓でも40～50%と高値を示す[11]. これは母体内で外界から保護されて急激に成長する胎児では，感染防御よりも十分な酸素供給が重要であるためと考えられる.

さらに胎児肝臓と骨髄の最も顕著な違いは，HSCの細胞周期と増殖性である. 骨髄のLTR-HSCはG1期が20%を占め，S/G2/M期が約5%であり，非常にゆっくりと細胞周期を移行している[12]. これは，成体骨髄HSCのうち一日あたり約8%が細胞周期に入り，99%のHSCが平均して57日で細胞周期を一周すると推定される. 骨髄HSCとはきわめて対照的に，E14.5肝臓に存在するHSCは40%がS/G2/M期にある[13]. こうした細胞周期の状態と一致して，HSCは胎児肝臓で盛んに増殖する. Emaら[14]は放射線照射マウスへの移植実験でE11からE18までの肝臓のHSC数を評価した. その結果，胎児肝にHSCがはじめて検出されるのはE12で，肝臓内のHSCはE16までに38倍に増加し，その後次第に減少していく. これらの結果は，胎児肝臓におけるHSCの増殖にはE14.5からE16.5の間にピークがあり，肝臓の代謝臓器としての成熟に伴って造血ニッチとしての機能は低下すると考えられる.

1）造血組織としての肝臓の構造

これまで成体の肝臓の構造に関しては盛んに研究されてきたが，造血器官としての胎生期肝臓の構造研究はあまりおこなわれなかった. Sasakiら[15]は光学顕微鏡と電子顕微鏡を用いてマウスE10からE19までの胎児肝臓の細胞構築を詳細に検討し，造血器官としての胎児肝臓を四つのステージにわけた(図❷).

ステージⅠはE10における造血の開始である. 肝臓原基は心周囲腔の下の横中隔のなかに存在する. 光学顕微鏡では類洞腔が広がってみえ，電子顕微鏡では血液細胞が一個ずつ肝芽細胞(Hepatoblast)の間や，内皮細胞と肝芽細胞の間に存在する. 類洞腔には未分化な血液細胞およびマクロファージが存在している. ステージⅡはE11からE12にあたり，肝臓内での造血領域の体積が拡大していくことが特徴である. E11では肝臓の34%をしめる造血部分が，E12では約70%まで急激に増大する. E11の血液細胞は，初期の肝細胞索において類洞壁または肝芽細胞の間に存在する. 血液細胞は盛んに増殖し，その結果初期の肝細胞索は著明に拡大して造血巣(hematopoietic foci)を形成する. E11まではマクロファージは初期の類洞の内側にみられるが，E12以降では造血巣のなかに頻繁に認められるようになる. このマクロファージ(とくにcentral macrophageとよばれる)を赤芽球が取り囲む赤血球小島(erythroblastic island)という特別な細胞構造[16]のなかで脱核した成体型赤血球産生が盛んにおこなわれる. この特徴的な構造は成体の骨髄や脾臓の赤脾髄においても認められる. ステージⅢはE13からE14にあたり，造血領域の体積がピークを迎え，肝臓全体の約3/4を占めるようになる時期である. E13には肝臓のほぼ全体が造血巣で占められるようになる. これらの造血巣は肝芽細胞によって取り囲まれ，赤血球小島はその中心に存在する. E15からE19のステージⅣは，肝造血が収束に向かい肝細胞の成熟が観察されることが特徴である. E14～15では，肝芽細胞が増殖し，造血巣は不規則な形態をとり始める. E15には，肝臓全体の60%程度に減少した造血巣において肝細胞の増殖が目立ってくる. E17～19では，造血巣は20%程度に減少する. 肝臓全体に拡張していた造血巣は，肝細胞索のなかに散在する丸く独立した形態となる. 肝細

胞は立方体状になり細胞質内にグリコーゲンを大量に蓄積するようになり，肝細胞索を形成し，類洞を含む血管系と胆管系からなる複雑な構造ができ上がる[17][18].

出生後は肝臓での造血はみられなくなるが，成体でも骨髄線維症など特殊な条件下では肝臓の造血支持能が再び認められるようになる．フェニルヒドラジン投与によって貧血を起こす髄外造血の実験モデルでは，造血巣が肝臓の類洞内に形成され，成体における肝造血は類洞内皮細胞によって支持されることが示唆される[19][20]．このように，胎児肝における造血と成体の髄外造血としての肝臓造血では大きな相違がみられる.

4．胎児肝臓造血の *in vitro* 培養システム

HSCの自己複製/分化を決定するのはHSCと造血ニッチとの相互作用であると考えられている．骨髄造血を解析するために，30年近く前にDexter[21]が骨髄ストローマ細胞を用いて造血を *in vitro* で再現する培養系を開発した．この培養系では，骨髄由来の単層の付着細胞によって数週から数ヵ月間に渡り造血が維持される．この培養系で，移植可能なHSCはある程度維持できたが[22]，HSC活性は培養期間中に著しく減少しており[23][24]，骨髄での造血を完全には再現していない.

骨髄が定常的な造血をおこなうのに対して，胎生期の肝臓では上述のようにHSCの急激な増殖を伴った一過性の造血をする．したがって，胎児肝臓の造血ニッチは骨髄とは異なったHSCの自己複製と分化を促進する分子を供給している可能性が考えられる．胎児肝臓の造血環境を構成する細胞には未分化な肝細胞，内皮細胞およびマクロファージなど多様な細胞を含んでいるが，骨髄の場合と異なり全体を容易に回収することができる．上述したように組織学的な検討では，造血巣を胎児期の肝芽細胞が囲んでいることから，これらが造血ニッチを形成すると考えられてきた.

胎児肝臓の造血環境を細胞レベルで解明する目的で，胎児肝臓細胞から細胞株を作成して *in vitro* で造血支持能を検討する研究がおこなわれてきた．なかでもWinemanら[25]は，E14肝臓にSV-40ラージT抗原の温度変異株をレトロウイルスで導入して不死化したストローマ細胞株を200クローン以上確立し，HSC支持能力を検討した[25]．そのなかでAFT024と名づけられた細胞株はE14肝臓および成体の骨髄から得たHSCを少なくとも4～7週間維持することができた[26]．コロニー形成法による造血前駆細胞は5週間の培養で100倍以上に増えていた[27]．放射線照射マウスキメラ法でこれらHSCはAFT024との培養後も培養前と同等のHSC活性を示した．AFT024細胞が胎児肝臓のどの細胞種に由来するのか不明であり，胎児肝臓のニッチを正確に再現しているのかという疑問はあるが，彼らはHSCを支持する分子をサブトラクションcDNAライブラリーの作成，cDNAマイクロアレイを用いて探索し，巨大なデータベースをインターネット上に公開している(The Stem Cell Database：http://stemcell.princeton.edu).

細胞を不死化する過程で，元来の性質が失われていくことをしばしば経験することから，われわれはE14.5肝臓細胞の初代培養系を確立した．この培養系でのおもな細胞集団は未分化な肝芽細胞で，肝臓特異的な蛋白質(アルブミンおよびαフェトプロテイン)を産生しているが新生児期および成体の肝臓で発現しているG6-Pase，TATなどの代謝酵素は発現していない．われわれはこの培養系でIL-6ファミリーのサイトカインであるオンコスタチンM(OSM)がグルココルチコイドとともに肝細胞の成熟を強力に誘導することを発見した[28]．OSMは代謝酵素の発現を促進するだけでなく，形態的な変化，グリコーゲン蓄積，解毒及び細胞間接着などの出生後の肝臓に特徴的な性状を誘導した．発生過程の肝臓では，OSMは$CD45^+$血液細胞で発現されており，肝細胞では発現してなかった．したがって，OSMは血液細胞と未分化な胎児肝細胞との相互作用に関与するサイトカインであると考えられる.

われわれはこの胎児肝臓細胞初代培養系を造血支持環境として用いてAGM由来の成体型HSCと共培養する系を確立した[29]．胎児肝臓の造血活性は胎児の発達に伴って変化するが，最も活発に造血がおこなわれているのはE14.5付近であるため(**図❷**)，この時期の肝臓細胞をストローマ細胞として選んだ．E11.5 AGMから精製したHSCを含む$CD34^+c\text{-}Kit^+$細胞は，E14.5肝臓細胞と共培養することによって1000倍以上の血球産生能を示した．AFT024ストローマ細胞株と異なり，われわ

れの共培養系ではAGM由来HSCからHSCを含む造血前駆細胞を著しく増加させることができる．コロニー形成法では，多分化能をもった造血前駆細胞をOSM添加時に150倍以上に増幅した．さらに放射線照射マウスへの移植実験ではHSC活性を少なくとも9倍以上の増幅を認めた．興味深いことにOSMはこの共培養系において血球産生には必須ではなかったが，移植可能なHSCの支持・増殖には必須であった．このようにわれわれのAGM/FL共培養系は，盛んな造血とHSCの増殖という胎児肝臓の二つの特徴を*in vitro*で再現するものである．さらにわれわれはこの初代共培養系を用いて，多様な細胞から構成される胎児肝臓から造血支持能をもつ細胞集団の同定を試みた．これまで胎児肝細胞を分離する適当な表面抗原は知られていなかった．そこで胎児肝細胞の遺伝子発現解析をおこない，膜蛋白質Dlk/Pref-1が肝芽細胞に特異的に発現しており，この発現によって肝芽細胞を分離できることを示した[30]．Dlk^{+}肝芽細胞の造血支持能をAGM/FL共培養系で検討したところ，胎児肝臓細胞全体を造血支持細胞とした場合と同等の造血支持活性が認められた[31]．これらの結果から胎児肝臓でのニッチを形成する主要な細胞は肝芽細胞であると考えられる．なおこのDlkは，AFT024にも発現が報告されているが，本培養系における造血支持活性は確認できていない．

おわりに

成体の骨髄造血が長期間にわたって安定的な血球産生をおこなうのと対照的に，胎児期の造血は，造血器官の移行を伴って造血細胞が質，量ともにきわめてダイナミックな変化をすることが特徴である．胎児肝臓は最初の成体型造血ニッチとされてきたが，さらに2005年にはGekasら[32]は胎盤も胎生期の重要な造血器官であることを報告した．彼らは，成体マウスへの移植実験で，E9.5からE18.5の卵黄嚢，AGM，胎児血，肝臓および胎盤の血液細胞のLTR-HSC活性を詳細に比較した．その結果，HSCはAGMとほぼ同時期のE10.5の胎盤でも検出されはじめ，その数は肝臓での増幅と同調してE11.5から13.5の間に急上昇し，以後肝臓のHSC数が最大になるE15.5にかけて急速に低下した．さらに興味深いことに，肝臓や胎児血中にまだHSCが検出されないE11.0において，胎盤では既にHSC活性が明確に認められた．これらの結果は，胎児肝臓だけでなく胎盤もHSCの増幅できる成体型造血ニッチとして機能することを示しているだけでなく，AGMと同様に胎盤で成体型HSCが独立に生じている可能性をも示唆する．このように胎児期には同時に複数の器官で造血がおこなわれるが，それらの相違点や生理的な意義はまだ明らかになっていない．

近年注目されている幹細胞療法や再生医療のなかで，骨髄や臍帯血のHSCの増幅はきわめて大きな意義をもつ．HSCの増幅が実質的に起こらない骨髄と異なり，生理的にHSCが増幅している胎児肝臓や胎盤の造血ニッチの解析によって，HSCの増殖・分化制御機構の解明が期待される．

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