

Embryonic and Fetal Hematopoiesis

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Basic Principles of Hematopoietic Development

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Acronyms and Abbreviations

AGM, aorta-gonad-mesonephros; Bmp, bone morphogenetic protein; 2,3-DPG, 2,3-diphosphoglycerate; E#, day of embryonic development, where the number indicates age of the embryo in days after conception; EPO, erythropoietin; fL, femtoliter; Gata-1, -2, and -4, GATA-binding proteins 1, 2, and 4; HSC, hematopoietic stem cell; Ihh, Indian hedgehog; IL, interleukin; NK, natural killer; P#, day of postnatal development, where the number indicates age of the neonate in days after delivery; pg, picogram; PU.1, purine box-binding transcription factor 1; Scl/Tal-1, stem cell leukemia/T-cell acute leukemia factor 1.

The complexities of hematopoietic system development have been highly conserved throughout vertebrate evolution. Understanding the embryonic and fetal origins of hematopoiesis provides important insights regarding the function of the adult hematopoietic system. Hematopoiesis in embryonic and fetal animals has been studied intensively for several decades as a model for hematopoietic progression in humans. Recent technical advances have allowed researchers to characterize the spatial and temporal relationships as well as the cellular and molecular mechanisms of hematopoietic development.

This chapter reviews the basic biology of hematopoietic development in the mouse (*Mus musculus*). This appraisal will emphasize hematopoietic events during the embryonic and fetal stages of development, but also will cover selected features of neonatal hematopoiesis.

BASIC PRINCIPLES OF HEMATOPOIETIC DEVELOPMENT

Cell Structure and Function

Blood cells produced at different stages of development differ in morphology and function. Thus, primitive ("fetal") cells fabricated early in gestation have markedly different properties from their definitive ("adult") counterparts produced during late gestation and in postnatal life. This principle has been characterized most completely in erythroid lineage cells. Primitive erythrocytes (RBCs) are formed in the yolk sac, whereas definitive RBCs are produced by the liver and later spleen and bone marrow. Primitive RBCs are nucleated in circulation until approximately day 12.5 (E12.5) of gestation, after which nuclei gradually become condensed before being shed between E14.5 and E16.5.³⁵

Enucleated primitive RBCs retain their large size and can remain in circulation until as late as postnatal day 5 (P5). Both primitive and definitive RBCs are released during most of the latter half of gestation (E10–E18), although the ratio shifts as time progresses from mainly primitive to mainly definitive RBCs.

Primitive and definitive RBCs can be distinguished by their size. The volume of primitive RBCs varies from 465 to 530 femtoliters (fL), which is approximately six times larger than that of definitive RBCs.³⁵ The hemoglobin content of primitive RBCs, 80–100 picograms (pg)/cell, also is nearly six times the amount found in definitive RBCs.³⁵ Both primitive and definitive RBCs have basophilic cytoplasm when first produced due to abundant rough endoplasmic reticulum, but basophilia recedes as maximal hemoglobin content is achieved.

Other hematopoietic lineages also differ in cell structure and function during the course of development. Primitive megakaryocytes from the yolk sac contain fewer nuclei of lower ploidy, are about half the size, and respond differently to cytokine stimulation relative to definitive megakaryocytes.⁴⁷ Primitive macrophages that originate in the yolk sac⁴² lack certain enzyme activities, are capable of division, and survive for extended periods compared to definitive monocyte-derived macrophages. These functional differences are related to the roles that the two cell populations appear to play. Primitive macrophages are the source for many tissue macrophages in embryonic through juvenile stages of development, whereas definitive macrophages are the source for circulating monocytes and resident macrophages characteristic of the adult immune system.

PRIMITIVE HEMATOPOIESIS

The processes that drive primitive and definitive stages of hematopoiesis as well as the events that regulate transition between the two stages are mediated by a constellation of factors.^{1,30,45,46} Cell adhesion factors, growth factors, and transcription factors that participate in this process often support differentiation of multiple hematopoietic cell types,^{10,29,36} and the dependence of a given cell lineage on any particular molecule may differ between primitive and definitive hematopoiesis.

Erythroid Cells

Hematopoiesis occurs at multiple sites within the embryo and in extraembryonic tissues. The first phase of blood cell production, referred to as primitive hematopoiesis, is responsible for producing blood elements during the earliest stage of embryogenesis. Primitive hematopoiesis takes place in the visceral yolk sac beginning at approximately E7.^{15,34} Thus, primitive hematopoietic cells are among the earliest distinct tissues to differentiate in the embryo. Formation of primitive cells declines rapidly after E11. The visceral yolk sac or extraembryonic splanchnopleure (the term for a

structure in which mesoderm and endoderm are directly apposed) arises from the migration of extraembryonic mesoderm streaming from the caudal primitive streak along the inner surface of the visceral endoderm. The mesodermal cells committed to initiate and support hematopoiesis have been termed hemangioblasts because the contiguity of primitive hematopoiesis and vasculogenesis in both space and time suggests that primitive hematopoietic and endothelial cells in the yolk sac share a common ancestor.^{1,9} Hemangioblasts arise as undifferentiated cells at the primitive-streak stage and commit to producing a particular cell lineage before blood island formation.^{34,44} These pluripotent cells also can differentiate into other mesenchyme-derived tissues.

Between E7.5 and E9, hemangioblasts form multiple aggregates termed blood islands.³⁵ Each blood island contains a central core of unattached inner hemangioblasts (hematopoietic progenitors) surrounded by a rim of spindle-shaped outer hemangioblasts (endothelial progenitors).¹⁵ Nucleated erythroid cells are first recognized in the cores of the blood islands at E8 and are evident circulating in the cardiovascular system starting at E8.25.¹⁸ At this stage embryonic erythroblasts enter the circulation, where they continue to divide until approximately E13.

The majority of cells produced during primitive hematopoiesis are of the erythroid lineage. Committed erythroid colony-forming cells arrive in the yolk sac at approximately E7.25. These cells expand until E8 and then differentiate into primitive erythroblasts; all colony-forming cells have regressed completely by E9,³⁴ which corresponds approximately to the earliest phase of definitive erythropoiesis. Primitive erythroblasts serve as the sole source of RBCs in the early embryo from E8 to approximately E10.5³⁴ and remain an important source of RBCs until E13. Thus, embryos with a developmental age between E8 and E11 that are anemic suffer from a defect in primitive erythropoiesis.^{26,38} Interestingly, seemingly profound defects in primitive hematopoiesis leading to persistent functional defects in adulthood may not elicit an aberrant hematologic profile in the embryo.

Other Cells

Recent studies suggest that other hematopoietic cell lineages also are generated in the yolk sac during this primitive stage of hematopoietic development. Primitive lymphoid precursors and even some adult stem cells evolve at E7.5 and subsequently seed other sites of hematopoiesis, including the aorta-gonad-mesonephros (AGM) region, umbilical vessels, and liver.⁴⁰ Primitive macrophages have been identified in the yolk sac by E8⁴–E9.³⁴ In vitro experiments have demonstrated that E7.5 yolk sac cells can give rise to functional megakaryocytic precursors by E10.5.⁴⁷ Many hemangioblasts actually serve as bipotent or oligopotent progenitors, including those capable of commitment to erythrocytic/myeloid,⁴ erythrocytic/megakaryocytic,²⁷ granulocyte/macrophage,³⁴ and lymphoid (B cell and T cell)/myeloid

lineages. Stem cells for mast cells have also been reported to arise in the yolk sac during primitive hematopoiesis.³⁴

DEFINITIVE HEMATOPOIESIS

The second stage of blood cell production, termed *definitive hematopoiesis*, is thought to arise primarily from the AGM.^{3,27} The AGM is an amorphous band of intraembryonic splanchnopleure that encompasses the dorso-medial wall of the abdominal cavity. The AGM domain is the main source of mesenchyme-derived, definitive hematopoietic stem cells (HSCs) that will serve the developing animal during late gestation and postnatal life. Initiation of definitive hematopoiesis ranges between E8.5 and E9.25, with definitive HSCs evident in the AGM by no later than E10. Peak production of HSCs in the AGM occurs between E10.5 and E11.5, at which time they comprise almost 10% of all AGM cells. Although controversial, some AGM-independent HSCs may also arise from the allantois, chorion, definitive placenta, umbilical arteries, and yolk sac. The actual contribution of these secondary sites to the overall HSC population has yet to be defined. However, the placenta appears to serve a particularly important role. The yolk sac also appears to be an essential secondary site because it is a source of multiple progenitor cell lineages and remains for at least a day after the AGM has halted HSC production.²⁸

Regardless of their original site of *de novo* synthesis, HSCs migrate to seed other locations that support definitive hematopoiesis: embryonic liver, followed by embryonic thymus, fetal spleen, and bone marrow (in that order). These latter destinations do not produce HSCs *de novo*, but rather contain niches suitable for expansion of newly arrived HSCs.³³ The suitability of such niches is controlled by specific characteristics of their stromal support cells.³³ The embryonic liver is colonized first, apparently because it shares many molecular and functional similarities with the yolk sac.³¹ It provides the major locus for definitive hematopoiesis from E12 to E16.³⁹ The HSCs enter the embryonic liver in several succeeding waves between E9/E10 and E12.¹² The first HSCs to enter the liver are pluripotent and can form any type of hematopoietic cell. Their first step in intrahepatic maturation is to commit to a more limited range of lineage options, typically as either an erythromyeloid progenitor or a common myelolymphoid progenitor.²² Definitive erythroid precursors mature and become enucleated within erythroid islands in the liver before entering the circulation.²⁷ Liver-derived myelolymphoid progenitors subsequently develop into bipotent cells (B cell and myeloid, or T cell and myeloid) before committing to produce a single-cell lineage.²² Some T-cell progenitors have a bipotent commitment to natural killer (NK) cell lineage. T-cell precursors destined for transfer to the embryonic thymus are produced even in athymic mice, indicating that the fetal liver may play a role in promoting early T-cell differentiation.^{20,21}

Embryonic thymus and fetal spleen are seeded either from the liver or AGM, or both, beginning about E13 for thymus and E15 for spleen. The thymus typically accepts only those HSCs that are committed to make T cells, whereas other multipotent myelolymphoid elements are directed to other sites.²⁰ The number of T-cell precursors in liver is abundant at E12, but decreases thereafter, whereas the population of intrahepatic B-cell progenitors exhibits a reverse trend.¹⁹ Most types of definitive hematopoietic cells in the spleen arise from precursor cells that commit to a specific lineage before leaving the liver. Multipotent HSCs entering the spleen cease proliferating and differentiate into mature macrophages. These cells may regulate intrasplenic erythropoiesis.

The bone marrow first receives HSCs from hepatic depots at about E16.^{39,45} Thereafter, the allocation of colony-forming hematopoietic precursors shifts from a primarily hepatocentric localization at E18 through a more dispersed distribution (bone marrow, liver, and spleen in approximately equal numbers) at P2 to a profile-favoring bone marrow and to a lesser extent spleen at P4 and after.⁴⁹ Thus, the bone marrow, liver, and spleen function cooperatively to regulate definitive hematopoiesis. While cooperating, each organ supports a molecularly distinct subset of hematopoietic progenitors.

Committed hematopoietic progenitors necessary to foster all lineages observed in adult animals arise during definitive hematopoiesis. The AGM-derived HSCs contribute to all major hematopoietic cell lineages. The HSC population from the placenta reportedly supports the genesis of erythroid, lymphoid (both B-cell and T-cell lineages), and myeloid elements. By comparison, the lineages sustained by yolk-sac-derived HSCs are limited to lymphoid and myeloid cells.⁴⁰ Whether or not progenitors for a given definitive cell lineage arising from distinct HSC populations exhibit different functional and molecular properties during late fetal and/or postnatal life has yet to be determined.

Late-stage embryos (E13–E15), fetuses (E16 to birth), and neonates which present with anemia are afflicted with a defect in definitive erythropoiesis. Abnormalities associated with this presentation include the total absence of definitive hematopoiesis,^{25,41} and an inability of progenitor cells to properly colonize intraembryonic sites of hematopoiesis. Multiple cell lineages may be affected; such a combined effect suggests that the hematopoietic defect occurs in a bipotent or multipotent stem cell rather than in one committed to forming a specific cell lineage.⁴³ Presentation with late-stage anemia also might result from a general delay in growth and development rather than a focused anomaly in the erythrocytic lineage.⁷

Young animals have circulating blood cell numbers that are different from that of adults.³⁹ RBC numbers are more than double between birth and young adulthood. Circulating leukocyte counts at birth are approximately 20% of adult levels before increasing to adult numbers by 6–7 weeks of age. Platelet counts in neonates are approximately one-third numbers.

HEMOGLOBIN SWITCHING

Primitive and definitive RBCs bear a battery of seven α - and β -globins, the mix of which varies with the developmental stage. The α -globins are encoded by three genes (ζ , α_1 , and α_2), whereas β -globins are encoded by four main genes ($\epsilon\gamma$, βH1 , βmin , and βmaj). The globins of a given type (e.g., α - or β -globins) typically exist as a series of closely linked homologous genes and related pseudogenes located on the same chromosome;^{16,24} mouse globin genes are carried on chromosomes 7 (β -globins) and 11 (α -globins). All seven mouse globin genes are transcribed during erythroid development, but the production of three— ζ , $\epsilon\gamma$, and βH1 —is limited to primitive RBCs.²³ A consequence of this limitation is that mouse β -globin genes, although closely related to human globins in most respects, do not follow the human pattern of upregulation in the sequence of their chromosomal arrangement.^{23,24}

The extent of individual globin gene expression and the blend of globin genes that are expressed vary over time. For example, enucleated primitive RBCs contain relatively more βmin than do definitive RBCs. At E11.5, βmin constitutes approximately 80% of the β -globin in circulation. This level is reduced by approximately 60% at birth. Primitive RBCs express increasing levels of adult globins as gestation progresses, whereas definitive RBCs harbor only the adult protein variants. This evolution indicates that the pattern of globin expression switches as the primitive RBCs are replaced by definitive RBCs. Molecular mechanisms which regulate the switching process are complex.¹⁷ The timing of this switch, between E10.5 and E12.5, coincides with the initial escalation in definitive erythropoiesis. Perturbed timing of this switch is a feature of some murine models of hematopoietic disease.⁶

Successful maintenance of the developing conceptus depends on preferential capture of oxygen in embryonic and fetal tissues. Therefore, primitive RBCs generally have a higher affinity for oxygen than do maternal RBCs, although domestic cats are an exception. This sequestration of oxygen is mediated by two primary mechanisms. The mechanism pertinent to the early embryonic period is the greater affinity of embryonic hemoglobin in primitive RBCs for oxygen relative to that of adult hemoglobin.² Alternatively, definitive RBCs in the late embryo and fetus possess a lower concentration of 2,3-diphosphoglycerate (2,3-DPG) than do maternal RBCs. Higher levels of 2,3-DPG facilitate oxygen release into tissues. After birth, levels 2,3-DPG content of RBCs rise to adult levels within 10–15 days.

MOLECULAR MECHANISMS REGULATING HEMATOPOIETIC DEVELOPMENT

A wide spectrum of growth factors, hormones, and transcription factors are required to specify the various stages of hematopoietic development in mammals. The entire meshwork responsible for directing any given

event has not been completely characterized. Shifting levels of several transcription factors have been shown to modify blood cell production. Insufficiencies in many of these molecules act by forestalling primitive hematopoiesis in the yolk sac. For example, genesis of erythroid precursors is impacted by deficits in GATA-binding protein 1 (Gata-1),¹³ shown in vivo to prevent erythroid maturation; Gata-2,⁴⁸ demonstrated in vivo to abort precursor expansion; and Gata-4,⁵ for which an in vitro shortage thwarts hemangioblast-mediated specification of blood islands and their associated vessels. These effects occur because the GATA consensus elements are critical regulatory regions in many erythroid-specific genes. All cell lineages are affected by stem cell leukemia/T-cell acute leukemia factor 1 (Scl/Tal-1).³⁸ Abnormal levels of transcription factors can also act later in gestation to disrupt definitive hematopoiesis. For example, purine box-binding transcription factor 1 (PU.1) is required for production of definitive (monocyte-derived) macrophages, but not their primitive (yolk-sac-derived) counterparts. This disparity in response is intriguing in that PU.1 is highly expressed during early hematopoiesis, but fluctuates in various cell lineages as time progresses.¹⁰ Normal genesis of many progenitor cells, including bipotent erythroid/megakaryocytic progenitors as well as B-cell and T-cell progenitors, requires that PU.1 levels be reduced, whereas production of myeloid progenitors necessitates an increase in PU.1.¹⁰

Secreted molecules also are important regulators of hematopoietic development during gestation. For example, erythropoietin (EPO) sustains both primitive and definitive erythropoiesis by stimulating proliferation and differentiation of immature primitive and definitive RBCs.²⁵ Reduction in EPO activity within the yolk sac greatly reduces the number of colony-forming cells and erythroblasts via excessive apoptosis. Thrombopoietin fulfills a similar function for megakaryocytes, although other cytokines (interleukin-3 [IL-3], IL-6) and growth factors (granulocyte-colony stimulating factor, stem cell factor) also are required.⁴⁷ Other ligand/receptor signaling pathways shown to affect hematopoietic development include the endoderm-derived molecule Indian hedgehog (Ihh)⁸ and bone morphogenetic protein 4 (Bmp4),¹¹ both of which participate in blood island production and vasculogenesis in the yolk sac. In general, secreted molecules act via their interaction with a specific transcription factor.

Cell adhesion molecules of the integrin family are essential for the proper migration of hematopoietic precursors. For instance, β_1 -integrins are essential if HSC are to reach the embryonic liver, and later the fetal spleen and bone marrow, at the appropriate developmental stages.³⁷ A loss of β_1 -integrins prevents adhesive interactions between HSCs and endothelial cells, thereby stranding the HSCs within vessels.³² Some integrins have functions in addition to their targeting role. For example, β_4 -integrins are required not only for correct homing but also for expansion and differentiation of erythroid and B-cell precursors in liver, spleen, and bone marrow. As with secreted factors, the activities of some

integrins relate more to late gestation and neonatal stages rather than earlier stages of hematopoietic development. This chronology has been documented for β_1 -integrin with respect to lymphoid and myeloid differentiation.¹⁴

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